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A RAPID METHOD OF TOTAL LIPID EXTRACTION AND PURIFICATION[1]

E. G. BLIGH AND W. J. DYER

Abstract

Lipid decomposition studies in frozen fish have led to the development of a simple and rapid method for the extraction and purification of lipids from biological materials. The entire procedure can be carried out in approximately 10 minutes; it is efficient, reproducible, and free from deleterious manipulations. The wet tissue is homogenized with a mixture of chloroform and methanol in such proportions that a miscible system is formed with the water in the tissue. Dilution with chloroform and water separates the homogenate into two layers, the chloroform layer containing all the lipids and the methanolic layer containing all the non-lipids. A purified lipid extract is obtained merely by isolating the chloroform layer. The method has been applied to fish muscle and may easily be adapted to use with other tissues.

Introduction

In the course of investigations concerning the deterioration of lipids in frozen fish, the need arose for an efficient and rapid method of total lipid extraction and purification. Furthermore, due to the highly unsaturated nature of fish lipids, the method had to involve only mild treatment so as to minimize oxidative decomposition and the production of artifacts. Several existing methods were considered but none was entirely satisfactory. The methods of Dambergs (1) and Folch *et al.* (2) were too time-consuming for routine investigations and since the former method entailed heating and evaporation it was considered unsuitable for lipid composition studies. The recent method of Folch *et al.* (3), which was published while this study was in progress, was more rapid than their previous method but still had the disadvantage of employing large and inconvenient volumes of solvent. The method used by Dyer and Morton (4) was rapid but extracted only a fraction of the total lipid.

The present paper describes a method whereby the lipids of biological materials can be extracted and purified in a single operation.

Mixtures of chloroform and methanol have had wide use as lipid extractants and examination of the chloroform–methanol–water phase diagram (Fig. 1) led to the following hypothesis. Optimum lipid extraction should result when

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Contribution from the Fisheries Research Board of Canada, Technological Station, Halifax, Nova Scotia.

the tissue is homogenized with a mixture of chloroform and methanol which, when mixed with the water in the tissue, would yield a monophasic solution. The resulting homogenate could then be diluted with water and/or chloroform to produce a biphasic system, the chloroform layer of which should contain the lipids and the methanol–water layer the non-lipids. Hence, a purified lipid extract should be obtained when the chloroform layer is isolated.

Procedure

Reagents

Methanol, absolute, analytical reagent; chloroform, analytical reagent.

Lipid Extraction and Purification

The following procedure applies to tissues like cod muscle that contain $80 \pm 1\%$ water and about 1% lipid. Each 100-g sample of the fresh or frozen tissue is homogenized in a Waring Blendor for 2 minutes with a mixture of 100 ml chloroform and 200 ml methanol. To the mixture is then added 100 ml chloroform and after blending for 30 seconds, 100 ml distilled water is added and blending continued for another 30 seconds. The homogenate is filtered through Whatman No. 1 filter paper on a Coors No. 3 Büchner funnel with slight suction. Filtration is normally quite rapid and when the residue becomes dry, pressure is applied with the bottom of a beaker to ensure maximum recovery of solvent. The filtrate is transferred to a 500-ml graduated cylinder, and, after allowing a few minutes for complete separation and clarification, the volume of the chloroform layer (at least 150 ml) is recorded and the alcoholic layer removed by aspiration. A small volume of the chloroform layer is also removed to ensure complete removal of the top layer. The chloroform layer contains the purified lipid.

For quantitative lipid extraction the lipid withheld in the tissue residue is recovered by blending the residue and filter paper with 100 ml chloroform. The mixture is filtered through the original Büchner funnel and the blendor jar and residue are rinsed with a total of 50 ml chloroform. This filtrate is mixed with the original filtrate prior to removal of the alcoholic layer.

Adaptation to Other Materials

The above procedure can be applied directly to any 100-g sample containing 80 g water. Many alterations of the procedure are permissible but it is imperative that the volumes of chloroform, methanol, and water, before and after dilution, be kept in the proportions 1:2:0.8 and 2:2:1.8, respectively. These ratios represent the total volumes present in the ternary systems, including the water present in the sample. Thus, in the extraction of materials that do not contain 80% water, either the size of the samples can be adjusted so that they contain 80 g water, or 100-g samples can be used and the volumes of chloroform and methanol changed to give the correct proportions. In cases where the moisture content is much less than 80% (e.g. fish meal), it is necessary to add distilled water. When material containing a large amount of lipid is used, or where the supply of material is limited, the size of sample can be reduced and the above solvent quantities scaled down to meet requirements.

Determination of Lipid Content

A portion of the lipid extract containing 100–200 mg lipid is evaporated to dryness in a tared flask and the weight of the lipid residue determined. Evaporation, facilitated by a stream of nitrogen, is carried out in a water bath at 40–50° C and the residue is dried over phosphoric anhydride in a vacuum desiccator. After weighing, a small volume of chloroform is added to each flask to detect the presence of non-lipid material (insoluble). If non-lipids are present, the chloroform is carefully decanted and the flask rinsed three times with chloroform. The dry weight of the residue is determined and subtracted from the initial weight. The lipid content of the sample is calculated as follows:

$$\text{Total lipid} = \frac{\text{weight of lipid in aliquot} \times \text{volume of chloroform layer}}{\text{volume of aliquot}}.$$

Experimental

Optimum Conditions for Lipid Extraction

The first objective was to find which of various mixtures of chloroform and methanol would yield quantitative extraction. The solvents were mixed in

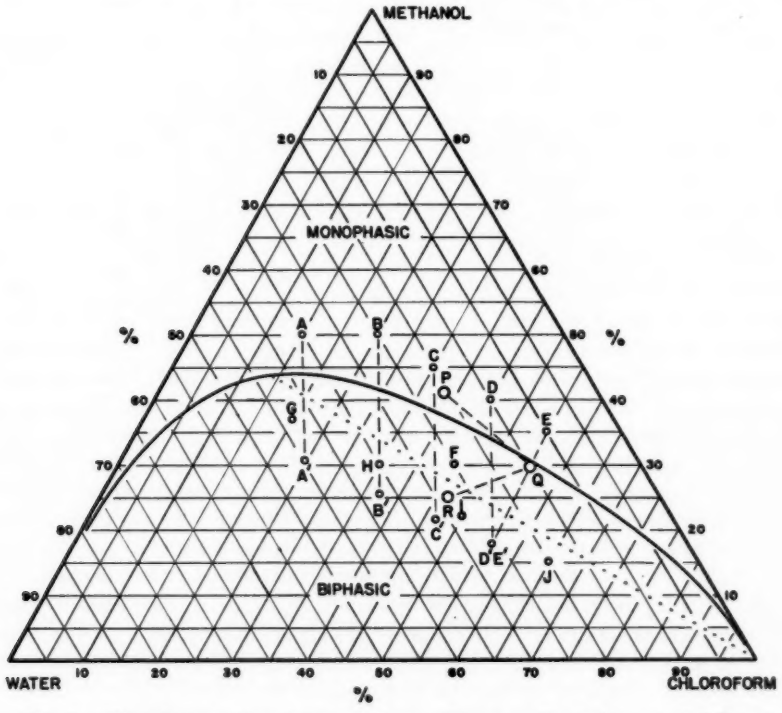

FIG. 1. Chloroform–methanol–water phase diagram, % (w/w) at 20° C (5). ..... "maximum chloroform tie-line" estimated from data for 0° C (6). - - - dilutions.

such proportions that, with the water of the samples, ternary systems were formed having compositions as marked on the phase diagram (Fig. 1, points A–J). Samples of minced cod muscle (100 g each) were homogenized with the extractants (Table I) and filtered. To the monophasic extracts, sufficient water

TABLE I

Lipid extracted from 100 g cod muscle by ternary mixtures of chloroform–methanol–water

Initial extraction mixture				Dilution solvents			
Ref. pt. in phase diagram (Fig. 1)	Chloroform, ml	Methanol, ml	Water,* ml	Chloroform, ml	Water, ml	Total volume, ml	Lipid in chloroform layer, g
A	23	144	80	40	87	374	0.31
B	54	202	80	104	154	594	0.56
C	94	228	80	166	184	752	0.63
D	162	270	80	285	228	1025	0.64
E	296	355	80	290	325	1346	0.62
F	97	121	80	†	†	298	0.56
G	50	174	160	†	†	384	0.32
H	54	87	80	†	†	221	0.32
I	96	80	80	†	†	256	0.40
J	175	76	80	†	†	331	0.41
P	100	200	80	100	100	580	0.70

*Including 80 ml from tissue.
†No additional solvents required to render system biphasic.

and chloroform were added to render the systems biphasic. The final compositions of these ternary mixtures are given by the points A′, B′, C′, and D′E′ in Fig. 1. The lipid contents of all chloroform layers were determined as above.

The results (Table I) confirmed the initial hypothesis in that more lipid was extracted by mixtures of the monophasic area of the diagram than by those of the biphasic area. Mixtures C, D, and E gave the highest values; however, the volumes of chloroform required for D and E were large and inconvenient. Therefore, the area around point C was considered most favorable.

A tie-line has been drawn in Fig. 1 which for the purposes of this paper has been called "the maximum chloroform tie-line". This tie-line was particularly significant in this study since the lower layers of ternary systems having compositions on or below it are practically 100% chloroform (6). Systems having compositions above this line have chloroform layers contaminated with methanol and water. This explains why the chloroform layer of the system represented by point F in Fig. 1 was the only one found to contain non-lipid.

Point P in the monophasic area of Fig. 1 was chosen as a convenient starting point for further investigation since the composition was equivalent with 100 ml chloroform, 200 ml methanol, and 80 ml water. The composition after adding another 100 ml chloroform is represented by point Q in the biphasic area of the diagram. Point R, which is below the maximum chloroform tie-line, is obtained by subsequent dilution with 100 ml water. This sequence was followed with cod muscle. The tissue (100 g) was homogenized with 100 ml chloroform and 200 ml methanol, filtered, and the residue rinsed with 100 ml chloroform in three portions. The pooled filtrates were mixed with 100 ml distilled water and the mixture was allowed to separate in a 500-ml graduated cylinder. Separation of the layers was rather slow but satisfactory. The

chloroform layer contained 0.70 g lipid, the highest yield obtained for this lot of cod muscle (Table I).

Further experimentation showed that the same result was obtained when dilution with chloroform and water preceded filtration. The chloroform had to be added before the water and each addition followed by blending. This resulted in a rapidity of separation of the layers and a yield of lipid which could not be obtained by adding the solvents in a different order.

The extraction of frozen samples did not significantly lower the final temperature of the extract; consequently it was unnecessary to thaw samples prior to extraction.

Substitution of glass fiber filter paper for Whatman No. 1 filter paper showed that with cod flesh there was no significant loss of lipid with the latter due to adsorption. Sintered glass filters also had no advantage over the regular filter except perhaps with materials such as fish meal.

Non-Lipid in Chloroform Layer

It was shown by three methods: (*a*) the determination of chloroform-insoluble material, (*b*) purification according to Folch *et al.* (2), and (*c*) purification according to Shorland *et al.* (7), that the lipid extracted by the above procedure contained no significant amounts of non-lipid material. The amounts of impurities, if present at all, were below the sensitivity limit of each of these methods.

Lipid in Methanol–Water Layer

The methanol–water layer was quantitatively removed and evaporated to less than 50 ml by distillation under reduced pressure at 40–50° C. The concentrate was quantitatively transferred to a separatory funnel and extracted four times with an equal volume of ethyl ether. Any emulsions were broken by centrifugation. The weight of lipid recovered from the pooled extracts was 8 mg. This loss, being only about 1% of the total lipid, was considered insignificant in most applications.

Lipid Remaining in Tissue Residue

Several procedures were employed to recover any lipid remaining in the extracted tissue. The most effective procedure involved re-blending the tissue residue and filter paper with 100 ml chloroform, followed by filtration and rinsing of the blendor jar and residue with a total of 50 ml chloroform. The recovered lipid weighed 40 mg, approximately 6% of the total extracted lipid. Re-extraction of the washed residue with chloroform–methanol–water did not yield any further amount of lipid. Thus, the initial extraction isolated approximately 94% of the extractable lipid.

The washed residue was further treated with 200 ml 6 *N* hydrochloric acid at 100° C for 90 minutes to establish whether there was lipid remaining which was bound in such a way that chloroform–methanol would not remove it. The liberated "lipids" were extracted from the digest according to A.O.A.C. specifications (8). The yield was 47 mg (Table II). At least part of this material may have originated from inositides (see (9)), the protein complexes

of which have been found to be resistant to chloroform–methanol extraction (2). Furthermore, the drastic acid treatment may have produced some fat-like material from non-lipid constituents of the muscle.

TABLE II

Grams of lipid extracted from 100 g cod muscle by four methods

Present method	Method of		
	Dambergs (1)	A.O.A.C. (9)	Folch *et al.* (3)
Sample I			
0.65	—	0.63	—
0.67	—	0.62	—
0.66	—	—	—
0.68	—	—	—
Sample II			
0.76	0.67	—	0.78
0.76	0.69	—	0.77
0.05*	0.07*	—	0.02*

*Lipid isolated from residue after digestion with hydrochloric acid.

Comparison with Existing Methods

Samples of well-mixed minced cod muscle (100 g each) were extracted using the described method and the methods of Dambergs (1), Folch *et al.* (3), and A.O.A.C. (8). The results are shown in Table II. The average lipid content of sample I as determined by the present method was 0.666% (standard deviation, 0.013), which was higher than the values obtained using the A.O.A.C. method (8). For sample II, which had a higher lipid content than sample I, the method of Folch *et al.* (3) and the present method gave yields which were not significantly different; however, the extract of the former method usually contained small amounts of non-lipid material. The values obtained using the method of Dambergs (1) were somewhat lower than those of the other methods.

The flesh of several species of fish (100-g samples) with different fat contents, and two lots of fish meal (10-g samples) were analyzed by the described method

TABLE III

Grams of lipid extracted from various samples using the present method and the method of Dambergs (1)

Sample*	Present method	Method of Dambergs (1)
Halibut fillets	0.75†	0.74
Cod fillets (pre-rigor)	0.76	0.68
Cod fillets (5 yr at −10° F)	0.56†	0.53
Cod fillets (18 mo at +10° F)	0.50†	0.49
Swordfish steaks	7.85†	6.51
Rosefish fillets	1.88	1.68
Plaice fillets	0.66	0.68
Fish meal (A)	0.34	0.30
Fish meal (B)	0.54	0.48

*All samples except the fish meals were 100 g; of the meals 10 g was used per extraction.
†Tissue residue was not washed.

and that of Dambergs (1). The results in Table III show that the new method for the most part extracted more lipid than the method of Dambergs (1).

Discussion

When the proper proportions of chloroform and/or water are added to a miscible chloroform–methanol–water mixture, the system separates into two layers. The proportions may be chosen in such a manner that the lower layer will be practically 100% chloroform and the upper layer nearly all methanol–water. This concept was applied to the extraction and purification of tissue lipids. It was found to be very effective; the miscible solvent mixture was an efficient lipid extractant which upon the addition of chloroform and water separated into two layers and in so doing separated the lipids from the non-lipids. The method which was developed is simpler and less time-consuming than procedures hitherto reported and is well adapted to routine lipid analyses, and also to lipid composition studies since only mild treatment of the samples is involved. For practical purposes, total lipid extraction was complete; slightly more fatty material could be isolated only after hydrochloric acid digestion. The separation of lipids and non-lipids was nearly quantitative. The results of lipid determinations on a sample of cod muscle were reproducible to within $\pm 2\%$ (standard deviation).

In some applications it may be unnecessary to wash the tissue residue since the washings contained only 6% of the total lipid and the lipid composition of the washings, as shown by free fatty acid and phospholipid determinations, did not appear to differ from that of the original extract.

Some chloroform is evaporated during filtration of the homogenate but this is not serious provided the decrease in volume is not too great.

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ANOMALOUS RESULTS IN THE PERIODIC ACID OXIDATION OF SERINE[1]

D. C. MORTIMER

Abstract

When serine-3-C^{14} was degraded with periodate at pH 5.5, the expected molar equivalents of carbon dioxide (carbon-1), formic acid (carbon-2), and formaldehyde (carbon-3) were obtained, but 15 to 31% of the C^{14} was recovered in the formic acid and the specific activity of the formaldehyde was reduced by a similar amount. These results combined with additional evidence from periodic acid uptake curves at different pH values, and the identification of glycolaldehyde isolated as the dinitrophenylhydrazone from the reaction mixture, showed that the serine–periodate reaction was more complex than has been assumed. A modification of the accepted mechanism was proposed to account for the anomalous results.

Introduction

The reaction between serine and periodic acid is often used in methods for estimating serine (1, 2). The products of the oxidation are formaldehyde, ammonia, and glyoxylic acid, and the amount of serine is calculated from the formaldehyde or ammonia recovered. The reaction has also been used to degrade C^{14}-labelled serine (3, 4, 5). In this application the glyoxylic acid product was further oxidized to formic acid and carbon dioxide, either as a separate step following the isolation of formaldehyde (3), or during the original periodic acid oxidation (4). In the latter case, the known acceleration of the oxidation by mildly alkaline conditions (6, 7) was applied. The mole of carbon dioxide recovered after 10 minutes' oxidation at pH 4 (7) was stated to arise from the oxidation of glyoxylic acid produced by the instantaneous cleavage of serine. The individual carbons of serine were therefore identified, since carbon-3 was recovered as formaldehyde, carbon-2 as formic acid, and carbon-1 as carbon dioxide.

In our study of the origin and fate of the C^{14} fixed in serine during photosynthesis, we tried several variations of the periodic acid reaction to degrade the molecule. Although we were eventually able to obtain quantitative recovery of each of the carbons, we could not get reproducible specific activities for carbons 2 and 3. The specific activities of carbon-1 and the *average* of carbons 2 and 3 were, however, reproducible. When the same degradation method was applied to radioactive glyceric acid samples, reproducible specific activities were obtained for each of the carbon positions.

These results suggested the need for a more detailed study of the periodic acid reaction with serine. This report presents evidence that an interchange of the carbon from positions 2 and 3 of serine occurs during the oxidation.

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Contribution from the Division of Applied Biology, National Research Council, Ottawa, Ontario.
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Experimental

Serine and calcium glycerate were obtained from Nutritional Biochemicals, and periodic acid from G. Frederick Smith. Sodium glyoxylate was prepared from tartaric acid by periodate oxidation (8). Serine-3-C^{14} was obtained from Tracerlab, Inc.

Time-course studies on periodate oxidation rates were conducted in 10 ml of solution containing about 0.4 mmole of serine, glyceric acid, or glyoxylic acid, and 1.0 mmole of periodic acid. Periodate remaining was determined by the arsenite–iodine titration (9).

The degradation of serine was modified to ensure complete oxidation by 2 equivalents of periodate. The carbon dioxide, formic acid, and formaldehyde from carbons 1, 2, and 3 respectively were quantitatively separated and recovered as follows: About 0.3 mmole of serine and 1.0 mmole of periodic acid were mixed in about 15 ml of solution in a 100-ml round-bottom flask. The flask was connected to a conventional alkali-charged bead tower through a reflux condenser. Sufficient alkali was added to the reaction flask through a side arm to raise the pH to 5.5. After 30 minutes the contents of the flask were acidified, and carbon dioxide free air was drawn through the assembly to sweep the carbon dioxide product of the reaction into the bead tower. The reaction flask was then fitted to a distillation assembly, the contents were neutralized, and the formaldehyde was steam-distilled into a receiver containing 2 ml of 1 *M* sodium bisulphite. The residual solution was acidified and the formic acid remaining was distilled into a receiver containing 1 mmole of alkali. It was necessary to reduce the volume in the distillation flask to less than 2 ml to ensure complete recovery of the formic acid.

The contents of the formaldehyde receiver were transferred back to a cleaned reaction flask connected to the aeration train. Three milliliters of saturated sodium dichromate and 5 ml of 10 *N* sulphuric acid were added and the mixture was heated under reflux for 30 minutes while carbon dioxide free air was drawn through the solution. The carbon dioxide formed by the oxidation of formaldehyde was collected in the bead tower as before. The contents of the formic acid receiver were treated in an identical manner.

The carbon dioxide absorbed by the alkali in the bead tower was precipitated as barium carbonate, and the residual alkali was titrated to obtain a measure of the amount of carbon dioxide produced. When the sample degraded was radioactive, the barium carbonate was collected, washed four times, and plated on weighed aluminum planchets.

An extensive series of recovery and control tests was carried out. When the complete degradation procedure was performed with serine replaced by formaldehyde, formic acid, or mixtures of formaldehyde and formic acid, formaldehyde was quantitatively recovered in the formaldehyde fraction, and formic acid in the formic acid fraction. There was no detectable oxidation of either substance to carbon dioxide. Glyoxylic acid carried through the same procedure gave molar equivalents of formic acid and carbon dioxide. The yields of formaldehyde, formic acid, and carbon dioxide derived from the oxidation of serine were, however, lower than those from the control runs.

The procedure was modified in many ways in attempts to improve the yields but these were not successful. The method adopted provided the best compromise between maximum yield and minimum time consumption. The entire procedure could be carried through in duplicate in 1 working day, thus avoiding the necessity of carrying over various fractions to another day. The probable reason for the low recovery with serine will be made apparent in the following discussion.

The radioactivity in the barium carbonate was measured with a Nuclear Chicago D47 Micromil-Window counter and scaler assembly. All activities were corrected to infinite thinness (10).

Results and Discussion

While the general pH dependence of the periodate oxidation is known, the curves shown in Fig. 1 provide some additional information. At pH 1 serine

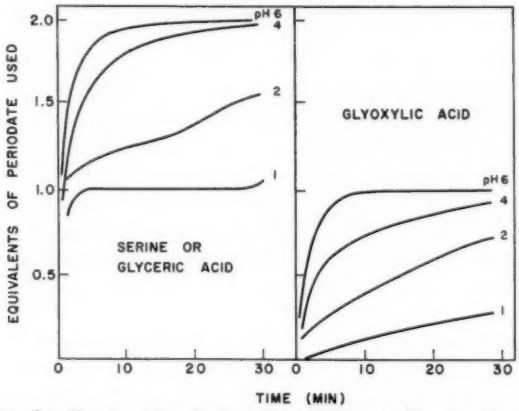


FIG. 1. Rate of utilization of periodic acid at different pH values by serine, glyceric acid, and glyoxylic acid. See text for details.

(or glyceric acid) consumes 1 equivalent of periodate very rapidly, but shows no further uptake for about 30 minutes. Under the same conditions, glyoxylic acid is slowly oxidized. As the pH of the solution is raised, the rate of oxidation increases. There is still a break in the curve at pH 2 with serine, but none with glyoxylic acid. At pH 6, the complete oxidation of the three substances to one-carbon products is accomplished within 10 minutes.

The consumption of 1 equivalent of periodate by serine is presumed to produce formaldehyde and glyoxylic acid. The oxidation curve should, therefore, show an additional periodate consumption equivalent to that observed for the oxidation of known glyoxylic acid. The absence of this extra oxidation at pH 1 suggests that glyoxylic acid is not immediately liberated, but may be linked in some form of complex with the periodate. At the higher pH values the rates of oxidation of both substances are too rapid to permit observation of any differences by conventional titration analysis.

When the degradation procedure was applied to a sample of serine-3-C^{14}, the product did not have the expected distribution of C^{14}. The formic acid (presumably carbon-2) was contaminated with C^{14} which must have come from carbon 3. The results of five analyses shown in Table I illustrate that the

TABLE I

Total recoveries and specific activities of the individual carbons from serine-3-C^{14} isolated as carbon dioxide following periodate degradation at pH 5

Run	Carbon-1		Carbon-2		Carbon-3		Average of $C_1+C_2+C_3$, c.p.m./mg
	%*	c.p.m./mg C†	%*	c.p.m./mg C†	%*	c.p.m./mg C†	
1	74	0	69	326	95	721	349
2	81	0	63	323	84	791	371
3	102	0	60	198	104	882	360
4	71	0	76	173	100	950	375
5	86	0	59	226	117	894	373

*Carbon dioxide recovered as per cent of the theoretical yield.
†Specific activity calculated from the recorded counts of plated barium carbonate corrected for self-absorption.

degree of contamination was variable, and did not show any dependence on the total recovery of carbon. If the C^{14} in formic acid had been due to oxidation of preformed formaldehyde, the yield of formic acid should have been high, and of formaldehyde, low. In addition, the loss of formaldehyde by oxidation would not have changed the specific activity of the formaldehyde recovered. Because the specific activity of the formaldehyde was lower when that for formic acid was higher, there must have been a transfer of carbon from the non-radioactive carbon-2 to carbon-3 equivalent to that in the reverse direction. An interchange of this type could occur if a 2-carbon intermediate like glycolaldehyde was formed.

To confirm that the serine-3-C^{14} was labelled exclusively in the position 3, a portion of the sample was treated with 1 equivalent of periodic acid (pH 1). The iodate produced was removed by precipitation with barium hydroxide. The dinitrophenylhydrazones (DNP's) of the oxidation products were precipitated by the addition of an acid solution of dinitrophenylhydrazine. The combined DNP's were taken up in ethyl acetate, glyoxylic acid DNP was separated by extraction into sodium carbonate solution, and formaldehyde DNP was recovered by evaporation of the ethyl acetate. When the recrystallized DNP's were assayed for C^{14} content, formaldehyde DNP was radioactive while glyoxylic acid DNP was not. The original serine-3-C^{14} was, therefore, correctly labelled and the observed interchange was associated with the oxidation reaction.

The yield of the DNP products was lower than expected, and there was evidence of a second DNP in the neutral fraction. It was distinguished from formaldehyde DNP by its deeper orange color, and could be separated from formaldehyde DNP by its poor solubility in ethanol. The unknown product was prepared in sufficient amount (yields in consecutive experiments were variable) to permit comparison of its absorption spectrum with those of authentic samples of formaldehyde DNP and glyoxylic acid DNP. In ethanol

solution the absorption spectra of all three substances were very similar throughout the visible and ultraviolet ranges, but in a solution of ethanol – ammonium hydroxide (3:2 v/v), interesting differences were apparent. As the solutions aged, the color of the neutral products changed from a wine red to brown and the color of the acid DNP from yellow to a deeper yellow. The absorption curves shown in Fig. 2 were obtained with solutions 5 minutes after mixing

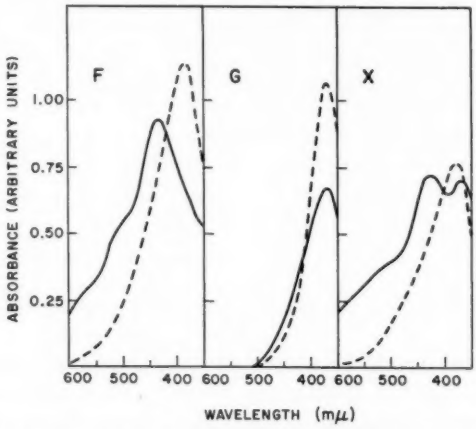
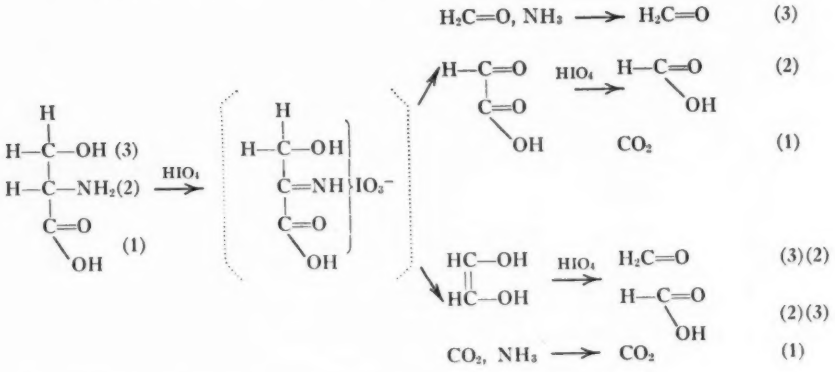


FIG. 2. Absorption spectra of 2,4-dinitrophenylhydrazones in ethanol – ammonium hydroxide (3:2, v/v); —— 5 minutes after mixing, – – – 5 hours after mixing.
F, formaldehyde DNP; G, glyoxylic acid DNP; X, unknown DNP and glycolaldehyde DNP.

and the same solutions 5 hours later. Each of the substances had a characteristic pattern and change in pattern due to the alkali treatment. It was established that dinitrophenylhydrazine and its alkali degradation and periodate oxidation products were not responsible for the unknown. When the recent studies of Arsenault (11) on methods for distinguishing between glycolaldehyde and glyoxal were brought to the attention of the author, a direct comparison of the spectra of the DNP's of these substances with that of the unknown was made possible. The comparison showed that the substance isolated had the same spectrum in ethanol – ammonium hydroxide solution as authentic glycolaldehyde DNP and that some glyoxal *bis*-DNP, probably derived from glycolaldehyde DNP, was also present. The evidence suggests, therefore, that glycolaldehyde is produced along with formaldehyde and glyoxylic acid during the periodic acid oxidation of serine.

That the periodic acid degradation of serine is not a simple glycol-type oxidation is apparent from the data presented above. The results of the serine-3-C^{14} analyses may be explained by assuming that the rate of oxidation at the C_1—C_2 bond occurs (at pH 5) at about the same rate as that at the C_2—C_3 bond. This would result in the formation of carbon dioxide (carbon-1) and a two-carbon fragment containing carbons 2 and 3 of the original serine in addition

to the expected products, formaldehyde and glyoxylic acid. Oxidation of the glycolaldehyde fragment could then yield formaldehyde and formic acid, but without specificity as to origin of the carbon, as illustrated below.



The complex nature of the oxidation reaction could also account for the reduced yields of products.

The periodic acid uptake curves at pH 1 and 2 support the proposed modification in the mechanism of oxidation if it is assumed that an increase in pH from 1 to 5.5 does not change the mechanism of the reaction. The presence of a complex could then exert a directing influence toward oxidation at the C_1—C_2 bond. The observation that glycolaldehyde was formed in a reaction at pH 1, and the additional note that the interchange was not prevented by allowing the degradation reaction to stand at pH 1 for 30 minutes before increasing the pH to 5.5, are both consistent with the suggested scheme. Aronoff (12) reported that it was necessary to allow a serine – periodic acid mixture to react for up to 24 hours to ensure a good yield of formaldehyde. We obtained similar results and could explain them only in terms of a complex, in view of the apparently rapid oxidation observed at pH 1.

The rate of periodic acid consumption by glyceric acid was, under the conditions of these experiments, the same as that for serine. Degradation of the glyceric acid samples referred to in the Introduction did not, however, result in an interchange like that observed with serine. Taken together with the data presented above, these observations support the view that the oxidation anomaly is associated with the vicinal hydroxy-amino structure. Although more work will be required to fill in the details of the mechanism, the present evidence leaves no doubt that isotope distribution data obtained by degrading radioactive serine, and perhaps other structures like glucosamine, with existing periodic acid methods must be interpreted with care.

Acknowledgment

I wish to acknowledge the valuable technical assistance given in this study by Miss Betsy L. Auld.

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PURIFICATION AND PROPERTIES OF PHOSPHOGLUCOSE ISOMERASE FROM ASPERGILLUS NIGER[1]

KARTAR SINGH[2]

Abstract

Phosphoglucose isomerase from *Aspergillus niger* was purified about fiftyfold by ammonium sulphate fractionation and treatment with calcium phosphate gel. The properties and kinetics of the enzyme are described. The enzyme resembled the muscle phosphoglucose isomerase in not requiring metal ions for activation but differed in showing no inhibition with phosphate. The enzyme showed maximum activity between pH 7.8 to 8.0 as compared with the optimum pH 9.0 shown by the muscle enzyme.

Introduction

The enzyme responsible for the catalysis of the reversible conversion of glucose-6-phosphate to fructose-6-phosphate was first demonstrated by Lohmann (1) with extracts from skeletal muscle and yeast. Somers and Cosby (2) found a similar enzyme in pea meal. The presence of phosphohexose isomerase in human erythrocytes has been reported (3). In addition to the specific phosphoglucose isomerase a phosphomannose isomerase was isolated from rabbit muscle (4, 5, 6). Although the existence of phosphoglucose isomerase in extracts of *Aspergillus niger* and *Penicillium chrysogenum* has been demonstrated (7, 8, 9, 10), no detailed study of this enzyme from molds has been made. Because of the important role of phosphoglucose isomerase in both the Embden–Meyerhof–Parnas glycolytic and the hexosemonophosphate oxidative pathways it was of interest to purify the mold enzyme and to study and compare its properties with those of the muscle enzyme. The present paper deals with the purification and properties of phosphoglucose isomerase of a citric-acid-producing strain of *A. niger*.

Materials and Methods

The experimental methods and materials used in this study were the same as described earlier (7, 11) except when otherwise indicated in the text. Barium salts of G6P* and F6P were commercial preparations from Schwartz Laboratories. Barium salt of M6P was a gift sample from Dr. H. A. Lardy. The barium salts were converted to potassium salts by treatment with the cation exchange resin Dowex 50 (12% cross-linked in H form) followed by neutralization with potassium hydroxide. *Zwischenferment* was purified from *A. niger* (12). Calcium phosphate gel prepared according to Keilin and Hartree (13)

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Contribution from the National Chemical Laboratory, Poona, India.
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*Abbreviations used: G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; M6P, mannose-6-phosphate; TPN, triphosphopyridine nucleotide; tris, aminotris(hydroxymethyl)methane; DPN, diphosphopyridine nucleotide.

was aged for 6 months before use. The stock gel preparation was diluted with acetate buffer, pH 6.0, to give a final concentration of 0.04 *M* acetate and 16 mg of calcium phosphate per ml.

The citric-acid-producing strain of *A. niger* used in the earlier studies (7, 8, 11) was employed; the methods used for growth of the organism, preparation of extracts, and determination of protein have been described in an earlier paper (11).

Measurements of pH were made with a glass electrode and optical measurements were carried out in a Beckman quartz spectrophotometer (model DU) using a 1-cm cuvette. Centrifugations were carried out in an International refrigerated centrifuge (model PR-1) and electrophoresis in a Tiselius electrophoresis apparatus (model 38, Perkin Elmer Corporation) with a Longsworth scanning device.

Glucose-6-phosphate was estimated by incubating the sample with purified *Zwischenferment* (200 μg), TPN (0.4 μmoles), magnesium chloride (45 μmoles), and glycylglycine (110 μmoles) in a final volume of 3 ml at pH 7.6 and measuring the total increase in light absorption at 340 mμ, the amount of G6P being equivalent to the TPN reduced. Aldolase was determined by the optical method of Warburg and Christian (14).

Phosphoglucose Isomerase Assay

Assay of phosphoglucose isomerase activity was based on that described by Slein (5), F6P formation being determined colorimetrically according to Roe (15). The test system contained 20 μmoles G6P, 0.7 ml 0.1 *M* tris buffer, pH 7.6, and enzyme in a final volume of 1 ml. The reaction mixture was incubated at 30° for 5 minutes and the reaction stopped by the addition of 6 ml 30% hydrochloric acid. Two milliliters of 0.1% resorcinol in 95% ethanol and 1 ml of water were added and the mixture was incubated for 10 minutes in a water bath maintained at 80°. The reaction mixture was cooled to room temperature and light absorption at 490 mμ was determined. The readings were corrected for G6P blank and compared with a fructose standard, the color due to F6P being taken as 65% of that given by fructose in the test. The amount of enzyme taken was so varied as to give an optical density reading of 0.2 to 0.4 under the assay conditions described above. Optical density was then proportional to the amount of enzyme (Fig. 1) and time of reaction.

One unit of phosphoglucose isomerase was defined as that amount of enzyme which catalyzed the formation of 1 μmole of F6P from G6P in 1 minute. Specific activity was defined as units of activity per milligram of protein.

Purification of Phosphoglucose Isomerase

Preparation of extracts of *A. niger* mycelium, fractionation with ammonium sulphate, protamine sulphate treatment for removal of nucleic acids, and calcium phosphate gel treatment were carried out in the same manner as described for the isolation of *A. niger* aldolase (11) up to the stage of gel absorption. These steps will therefore be described here very briefly. Whereas aldolase was completely adsorbed on the gel most of the phosphoglucose

isomerase activity remained in the supernatant. The amount of gel required varied slightly from batch to batch and was determined in preliminary experiments with small samples of the enzyme. The gel supernatant was further fractionated with ammonium sulphate. The purification procedure to which each batch was submitted was as follows:

Step 1.—Frozen mycelium (8 kg fresh weight) was extracted with 8 liters of 0.01 *N* ammonia solution. The extract (pH 6.3 to 6.5, fraction A) was brought to 0.8 saturation with ammonium sulphate and filtered on large Büchner funnels. The precipitate was suspended in 1500 ml of ice water and the solution was brought to 0.5 saturation with ammonium sulphate. The precipitate was discarded and the supernatant was brought to 0.8 saturation and centrifuged. The precipitate obtained between 0.5 and 0.8 ammonium sulphate saturation was dissolved in 260 ml of ice water and brought to pH 7.2 with 2*N* ammonia solution (fraction B). Saturated ammonium sulphate solution, pH 7.2, was added to the enzyme solution and the precipitate obtained between 0.6 and 0.8 saturation was collected by centrifugation and dissolved in ice water. The above fractionation between 0.6 and 0.8 saturation was repeated once more. The precipitate (fraction C) was found to be stable at $-20°$ for several months.

Step 2.—Fraction C was dissolved in about 500 ml of ice water and the pH adjusted to 5.8 to 6.0 with acetate buffer. The solution was then treated with sufficient protamine sulphate till no precipitate appeared on further addition of protamine sulphate (300 ml of 1% solution, pH 5.8). The precipitate obtained was discarded and the supernatant treated with 160 ml of calcium phosphate gel to completely adsorb aldolase. The gel supernatant was brought to 0.75 saturation with ammonium sulphate and the precipitate was dissolved in about 100 ml of ice water, reprecipitated at 0.75 saturation three times in order to remove residual protamine sulphate, and dissolved in 45 ml of ice water (fraction D). Saturated ammonium sulphate solution was added to the enzyme solution. The fraction obtained between 0.58 and 0.72 saturation was dissolved in about 15 ml of ice water and the pH adjusted to 7.6 with 2 *N* ammonia solution (fraction E).

Step 3.—All further fractionations were carried out at pH 7.6 with saturated ammonium sulphate solution adjusted to pH 7.6 (1.3 ml of 2 *N* ammonia solution per 100 ml saturated ammonium sulphate solution). Fraction E was brought to 0.7 saturation with ammonium sulphate and the precipitate obtained was dissolved in 15 ml of ice water (fraction F). Fraction F was further fractionated with ammonium sulphate and the precipitate obtained between 0.60 to 0.68 saturation was collected (fraction G). This fraction had the highest specific activity and was used for all the studies reported here. Attempts to further purify the enzyme by a second gel treatment followed by ammonium sulphate fractionation at different pH's were unsuccessful.

Results

Results of a typical purification procedure are given in Table I. Similar results were obtained with four other batches of mycelium. The specific

activity of the purest preparation obtained from the different batches varied between 60 and 75 units per mg protein.

TABLE I

Purification of phosphoglucose isomerase

Enzyme fraction	Volume, ml	Activity, units/ml	Total activity, units	Protein, mg/ml	Total protein, mg	Specific activity, units/mg
A Extract	11,000	10	110,000	7.0	77,000	1.43
C $(NH_4)_2SO_4$ fraction 0.6–0.8*	500	110	55,000	8.0	4,000	13.8
D Gel supernatant precipitated 4 times	48	820	39,360	41.0	1,968	20.0
E $(NH_4)_2SO_4$ fraction 0.58–0.72	17	1,418	24,106	43.8	745	32.1
F $(NH_4)_2SO_4$ fraction 0.70	15	1,170	17,550	30.0	450	39.0
G $(NH_4)_2SO_4$ fraction 0.6–0.68	13	740	9,820	10.6	138	70.0

NOTE: 8 kg of *A. niger* mycelium was used.
*These figures refer to the degree of saturation with $(NH_4)_2SO_4$.

Electrophoresis.—The purified enzyme was precipitated at 0.75 ammonium sulphate saturation and dissolved in a small quantity of buffer which was used for electrophoresis. The solution was dialyzed with stirring at 0° against three changes of the same buffer for 24 hours. Electrophoresis was carried out in phosphate buffer (ionic strength 0.1, pH 7.6; protein 0.6%). Electrophoresis for 240 minutes showed that at least three electrophoretically distinguishable components were present in the preparation.

Properties.—Aqueous solutions of purified *A. niger* phosphoglucose isomerase were clear and colorless. The solubility of the enzyme in ammonium sulphate solutions was similar to that of the muscle isomerase (5). Ammonium sulphate precipitates of the enzyme did not show any loss in activity when stored for 3 to 4 months at −20°. Solutions in water, phosphate buffer, or tris buffer, pH 7.6, retained full activity for several days when stored at 0 to 5°; a 0.024% enzyme solution lost 10% of its activity in 10 minutes at 40° and was completely inactivated in 5 minutes at 50°. The enzyme was stable to prolonged dialysis at 0 to 5° against 0.05 *M* phosphate, pH 5.5, or 0.05 *M* tris buffer, pH 7.6.

Specificity.—The purified isomerase was found to be specific for G6P, no F6P being formed when 5 μmoles M6P were incubated with 30 μg of enzyme for 20 minutes. The enzyme was also free of *Zwischenferment*, phosphogluconic dehydrogenase, and aldolase.

Equilibrium constant.—Glucose-6-phosphate was incubated with a large excess of the enzyme at 30° and pH 7.6. The equilibrium constant was determined by estimating G6P and F6P in the reaction mixture after the equilibrium had been attained. The reaction was stopped with hydrochloric acid and G6P and F6P were determined in aliquots of neutralized reaction mixture. The average ratio of G6P to F6P concentration, at equilibrium was 2.43 (Table II).

TABLE II

Equilibrium constant

Expt. No.	Initial G6P, μmoles	Final G6P, μmoles	Final F6P, μmoles	Ratio, G6P/F6P
1	10.15	7.33	3.08	2.36
2	20.30	14.26	5.91	2.40
3	20.30	14.54	5.78	2.52
4	10.15	7.31	2.99	2.44

NOTE: The test system contained 0.9 mg phosphoglucose isomerase, 70 μmoles tris, and the amount of G6P indicated below in 1 ml final volume at pH 8.1, temperature 30°, time of incubation 30 minutes.

Effect of pH.—The pH activity curve for phosphoglucose isomerase is shown in Fig. 2. Phosphate, veronal, or tris buffers, 0.1 *M* at the same pH, gave identical values for enzyme activity.

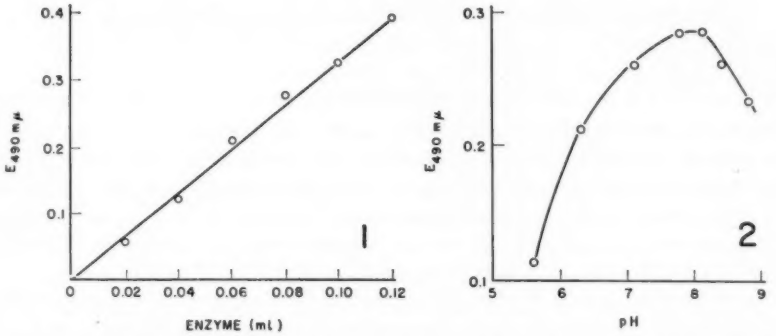

FIG. 1. Effect of enzyme concentration on phosphoglucose isomerase activity. Reaction mixture containing 20 μmoles G6P at pH 7.6 and varying amounts of enzyme solution (24 μg/ml), incubated for 5 minutes at 30°.
Ordinate: Absorption at 490 mμ.

FIG. 2. Effect of pH on phosphoglucose isomerase activity. Phosphate buffers 0.1 *M* for pH's 5.5 to 7.0 and tris buffers 0.1 *M* for higher pH's. Reaction mixture contained 20 μmoles G6P and 2 μg enzyme; incubation time, 5 minutes at 30°.
Ordinate: Absorption at 490 mμ.

Effect of temperature.—The effect of temperature on enzyme activity is shown in Table III. Glucose-6-phosphate was incubated with tris buffer at

TABLE III

Effect of temperature on phosphoglucose isomerase activity

Temperature	Change in optical density at 490 mμ (E)	Protein, units/mg
20°	0.217	39
26°	0.325	58
30°	0.388	69
40°	0.470	84

NOTE: The enzyme activity was determined by the routine assay method using 2.7 μg of the purified enzyme for each test.

the required temperature and the reaction was started by the addition of 0.1 ml of the enzyme.

Effect of inhibitors.—Unlike the muscle phosphoglucose isomerase the *A. niger* enzyme was not inhibited by phosphate. Fluoride (0.01 *M* in the presence of phosphate), iodoacetate (0.01 *M*), and cysteine (0.005 *M*) were without effect on isomerase activity. The activity was also unaffected by glucose (0.03 *M*), TPN (0.3 mg), or DPN (0.4 mg) in the test system.

Effect of metal ions.—The enzyme, even after prolonged dialysis against tris buffer, did not require metal ions for activation. Magnesium, calcium, manganous, or ammonium ions in 0.005 *M* concentration did not have any effect on the enzyme activity. Magnesium ions at 0.05 *M* concentration caused 7% inhibition.

Discussion

Phosphoglucose isomerase purified from *A. niger* had a specific activity of 75 at pH 7.6 and 30° as compared with the specific activity of 50 (recalculated in our units) at pH 9.0 and 30° for the muscle enzyme (4). The *A. niger* enzyme was still impure as shown by electrophoresis. It resembled the muscle enzyme in not requiring metal ions or other cofactors for activity but unlike the muscle enzyme it was not inhibited by phosphate ions. As compared with the muscle enzyme the inhibition due to 0.04 *M* magnesium was much less for the *A. niger* phosphoglucose isomerase. The ratio of G6P to F6P concentration at equilibrium was 2.43 for the *A. niger* enzyme while that reported by Slein (5) for the muscle enzyme was 2.13.

Tsuboi, Estrada, and Hudson (16) have recently reported the purification of phosphoglucose isomerase from human erythrocytes. Calculation from the turnover rates indicates that their enzyme preparation is 3 to 4 times purer than the *A. niger* enzyme.

Acknowledgment

The continued interest and advice of Dr. V. Jagannathan is gratefully acknowledged.

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STUDIES ON WHEAT PLANTS USING CARBON-14 LABELLED COMPOUNDS

X. THE INCORPORATION OF GLUTAMIC ACID-1-C^{14} [1]

W. B. McConnell

Abstract

Glutamic acid-1-C^{14} was injected into the top internode of wheat stems at a stage of growth when kernel development was rapid (71 days after seeding). The plants were harvested 31 days later when they had matured and the incorporation of carbon-14 studied. About one-third of the carbon-14 administered was found in the upper portions of the mature plants, much of the remaining radioactivity having apparently been respired. About 85% of the carbon-14 recovered was found in the kernel. The protein fractions of these were most radioactive, but an appreciable amount of carbon-14 also appeared in the starch. Glutamic acid had the highest specific activity of the amino acids isolated from the gluten, but proline and arginine were also strongly labelled. Since these three amino acids were labelled predominantly in carbon-1 their close metabolic relationship in the wheat plant seems probable.

Introduction

In earlier studies on the fate of certain carbon-14 labelled compounds when injected into stems of wheat plants (1, 2) a parallelism was consistently observed in the labelling of glutamic acid, proline, and arginine isolated from proteins of mature kernels. For example, when acetate-C^{14} (1) was injected, glutamic acid of comparatively high specific activity was recovered, the proline and arginine being the amino acids of next highest carbon-14 content. These observations are in accord with the commonly held concept of proline and arginine biosynthesis from glutamic acid, as known to occur in microorganisms (3) and in mammals (4).

A more direct demonstration of this interrelationship is now presented by the finding that glutamic acid-1-C^{14} does indeed lead to the formation of wheat kernel proteins containing proline and arginine labelled predominantly in the carboxyl group.

Experimental Methods

Thatcher wheat was seeded outdoors on May 23, and 71 days later the plants were injected with 0.2 ml of aqueous glutamic acid-1-C^{14} (0.63 mg glutamic acid, 10 μc of carbon-14 (1)). The injections were made into the hollow of the top internode on one tiller of each of eight different plants. The labelled tillers were harvested on September 2 (31 days after injection), and kernels from the seven plants which appeared to have matured normally were combined, dried, and ground to pass a 40-mesh screen to yield 6.12 g of meal. Similarly, ground

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samples of chaff and stem portion above the top node weighing 1.91 and 2.60 g, respectively, were collected from the seven plants.

Meal from the kernels was separated into fractions designated as starch, gluten, salt-soluble protein, ether-soluble material, and bran (or residue) according to the previously adopted technique (5) and the specific activity of each fraction was determined after combustion to carbon dioxide (1, 6). Some amino acids were isolated (7) from acid hydrolyzates of the gluten and each of these was decarboxylated with ninhydrin for separate recovery of carbon-1 (8). Specific activities of amino acids and of the carboxyl carbons were also determined by counting the carbon as carbon dioxide-C^{14}. Methods for further degradation of glutamic acid, serine, and alanine have been previously described (9, 10, 11, 12).

Results and Discussion

Upper portions of the mature plants contained about 35% of the carbon-14 injected and, since the rest of the plant had very low specific activity, extensive losses of carbon-14 from the plant are indicated (Table I). The kernels comprised most of the weight of the parts collected and since they were also of the highest specific activity they accounted for about 85% of the carbon-14 recovered.

TABLE I

Distribution of carbon-14 in plant parts

Plant part	Weight, g/plant	Specific activity, mμc/mmole CO_2	C^{14} content,* μc/plant
Kernel	.874	96	3.00
Chaff	.275	29	.29
Stem	.372	16	.22
		Total	3.51

*Glutamic acid-1-C^{14} injected 10 μc/plant. An undetermined amount of tracer was lost during injection of one of seven plants.

Proteins (Table II) were the most radioactive of the kernel fractions isolated, but both the starch and the residue had evidently incorporated considerable

TABLE II

Distribution of C^{14} in kernel components

Fraction	Yield, g	Specific activity, mμc/mmole CO_2	Total C^{14}, μc	C^{14}, % of total in meal	C^{14}, % of total injected
Meal	1.00	96	3.5*	100	31
Alkali-soluble protein	.04	95	.11	3.1	1.0
Gluten	.12	230	1.18	33.8	10.3
Starch	.56	59	1.71	48.8	15.0
Bran	.10	55	.27	7.8	2.4
Lipid	.05	14	.04	1.1	.4

NOTE: Yield of wheat kernels was 0.87 g/head. Therefore 11.4 μc was administered for each gram of wheat kernels collected.
*Estimated by plate counting sample of meal.

carbon-14. These data are in accord with the ideas regarding the active role of glutamic acid in plant metabolism. Ether-soluble material was low in carbon-14, and some radioactivity was included in aqueous extracts not examined.

Table III lists the relative specific activities of the amino acids of gluten (glutamic acid taken as 100). The observed specific activity of glutamic acid

TABLE III

Relative specific activity of amino acids of gluten (glutamic acid = 100)

Amino acid	Relative specific activity		Amino acid	Relative specific activity	
	Total	Carbon-1		Total	Carbon-1
Glutamic acid	100	434	Aspartic acid	14	16
Arginine	52	220	Threonine	12	19
Proline	48	194			
			Leucine	8	11
Glycine	28	32	Isoleucine	8	25
Serine	24	30	Valine	4	7
Alanine	19	25			
			Histidine	6	7
Tyrosine	16	16	Lysine	3	7
Phenylalanine	13	19	Methionine	9	19

NOTE: Tabulated values are the specific activities expressed as mμc/mmole CO_2 divided by 4.47 mμc/mmole CO_2.

was 447 mμc per millimole CO_2 and hence tabulated values were obtained by dividing the observed specific activities by the factor 4.47 mμc/mmole CO_2. The relative specific activity of the wheat meal was 21.5, gluten 51.5, and the starch 13.2. Glutamic acid was clearly much more radioactive than any of the other amino acids but the specific activity of arginine and proline was about one-half that of glutamic acid. Short-chain amino acids, glycine, serine, and alanine, were next in order of specific activity but appreciable radioactivity was also found in aromatic amino acids. Complete degradation of serine and alanine showed that, in each of the amino acids, carbons 2 and 3 were about equally labelled. Glutamic acid was evidently not as effective for labelling aspartic acid or threonine, the latter having specific activities approximately the same as the starch. Branched-chain amino acids and the basic amino acids, histidine and lysine, were also low in carbon-14.

Carboxyl carbons of the amino acids were in general somewhat more radioactive than the amino acids as a whole. However, differences were small except for glutamic acid, proline, and arginine in which there was a pronounced tendency for carbon-14 to appear in position 1. Figure 1 gives the ratios of the specific activity of carbon-1 to the calculated mean specific activity for remaining carbons of amino acids with relative specific activities above 10.

The results are in accord with the idea that proline and arginine are derived from the carbon skeleton of glutamic acid. Such a relationship has been well established for many living systems and its occurrence in maturing wheat plants was to be anticipated. However, these findings also demonstrated clearly the usefulness of the present procedure for revealing metabolic interrelationships between amino acids and probable precursors. Thus, the specifically labelled amino acids were isolated from the protein of wheat plants that had been left in

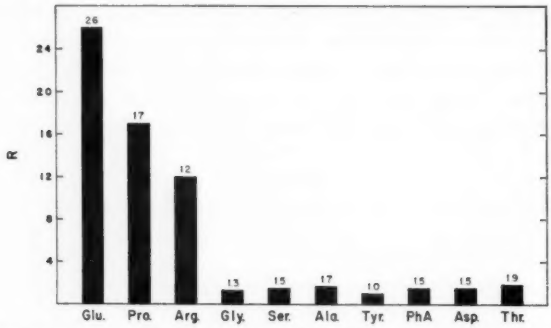

FIG. 1. Comparison of specific activity of carbon-1 of amino acids with average specific activity of remaining carbons. R = specific activity carbon-1/average specific activity remaining carbons. Glu. = glutamic acid, Pro. = proline, Arg. = arginine, Gly. = glycine, Ser. = serine, Ala. = alanine, Tyr. = tyrosine, PhA = phenylalanine, Asp. = aspartic acid, Thr. = threonine.

the field for 31 days after injection of tracer. It seems probable, therefore, that the radioactive amino acids, once formed, are effectively, and perhaps rapidly, utilized during the stages when kernel protein is accumulating and that, after being incorporated into "storage" proteins, they remain substantially unchanged.

Acknowledgments

The author is indebted to Dr. E. Bilinski for contributions to this work, to Mr. M. Mazurek for able technical assistance, and to Mr. J. Dyck for determinations of specific activity.

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LIPASE ACTIVITY IN LINGCOD MUSCLE PREPARATIONS[1]

J. D. Wood

Abstract

A lipase has been isolated and partially purified from the muscle of the lingcod (*Ophiodon elongatus*). Both monoglycerides and triglycerides are hydrolyzed but methyl acetate and ethyl acetate are attacked extremely slowly, if at all. The lipase is inhibited by *p*-chloromercuribenzoate but not by ferricyanide, cyanide, or azide. The pH of optimum activity is 8.2. The enzyme is unstable in aqueous solution, but can be stored at −20° C for several weeks without loss of activity.

Introduction

Frozen fish deteriorates gradually during storage. One aspect of the deterioration is the off-flavor produced by fat spoilage. The most well-known type of fat spoilage is oxidative rancidity. There is, however, a second type, namely, hydrolysis of the fat to produce free fatty acids. Brocklesby (1) showed that there was a gradual increase in free fatty acids during storage of various species of Pacific salmon. Mel'nikova and Khalina (2) observed similar changes in frozen mackerel. More recently Dyer and his co-workers (3, 4, 5) investigated the hydrolysis of lipids in frozen fillets of plaice, halibut, rosefish, and cod which were stored at different temperatures. Fatty acid formation was also observed by Cardin and Bordeleau (6) to occur during the process of salting codfish. At the present moment there are two different theories as to the mechanism by which the fatty acids are formed. Dyer and co-workers believe that the acids are released from lipids by the action of a lipase present in the fish muscle. This view is supported by Cardin and Bordeleau. Lovern *et al.* (7) however favor the theory that the formation of fatty acids is due to bacterial action.

Lipase activity has been studied extensively in material from various sources such as hog pancreas (8), wheat germ (9), and *Fusarium lini* Bolley (10). Several methods have been employed to purify the enzyme including precipitation by ammonium sulphate (9), magnesium sulphate (11), and acetone (10). There are also numerous reports on lipase activity in fish (e.g. 12, 13, 14) but the preparations used were crude and in most cases tissues other than the muscle were employed.

Because of the lack of information on lipase activity in fish muscle and in view of the importance attached to the spoilage of fish during storage, it was decided that an investigation into the occurrence and properties of lipase enzymes in fish muscle would yield useful information. This paper presents the results of such an investigation.

Methods

Lingcod Muscle

The tissue employed in the experiments was obtained from lingcod (*Ophiodon elongatus*) which were obtained fresh. The fish were either used at once or

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Contribution from the Fisheries Research Board of Canada Technological Station, Vancouver, British Columbia.

were frozen and stored at −20° C until used. The enzyme was found to be still active after several weeks at −20° C.

Measurement of Lipase Activity

Two methods were employed for determining the activity. One method was that first proposed by Rona and Lasnitzki (15), which was based on the manometric determination of carbon dioxide evolved from a bicarbonate buffer as a result of the liberation of fatty acids by the enzyme. The other method employed was the titration of the fatty acids liberated.

1. Manometric method. The incubations were carried out in the conventional Warburg apparatus at 25° C and under an atmosphere of 95% nitrogen and 5% carbon dioxide. The total volume of the flask contents was 3.0 ml. The reaction was carried out in 0.025 *M* $NaHCO_3$ unless otherwise stated. This gave a pH of 7.54 when in equilibrium with the gas phase (16). The substrate, dissolved in 0.025 *M* $NaHCO_3$, was placed in the side arm of the vessel. One milliliter of enzyme solution and 1.0 ml 0.05 *M* $NaHCO_3$ were placed in the main compartment. Substrates which were not sufficiently soluble were homogenized in polyvinyl alcohol prepared by the method of Fiore and Nord (17), and which contained 0.025 *M* $NaHCO_3$.

2. Titrimetric method. The technique employed was that reported by Fiore and Nord (17) with the following two alterations. The temperature employed was 25° C and the McIlvaine buffer was replaced by 0.1 *M* tris(hydroxymethyl)aminomethane because of the high pH range (7.8 to 9.0) required during the experiment.

Protein Estimation

This was performed using either the method of Kingsley (18) as adapted by Snow (19) or that of Lowry *et al.* (20).

Isolation and Purification of the Enzyme

A weighed quantity of lingcod muscle, either fresh or frozen, was homogenized in three volumes of ice-cold, distilled water and centrifuged at 20,000 *g* for 20 minutes. The supernatant fluid was collected. In this paper it will be designated muscle extract.

The extract was treated with $MgSO_4$ solution (60 g/100 ml) at 0° C and stirred for 10 minutes. The mixture was centrifuged at 20,000 *g* for 20 minutes and the protein precipitate was resuspended in ice-cold, distilled water and made up to a volume equal to one-fourth of that of the original extract. The suspension

TABLE I

Purification of lingcod muscle lipase

Preparation	μl CO_2 evolved/ hour/mg protein N	Activity recovered (%)	Purification
Muscle extract	91	100	
Precipitated with $MgSO_4$	945	18	10.4
Precipitated twice with $MgSO_4$	912	5	10.0

NOTE: Substrate was 8.4×10^{-2} *M* triacetin.

was filtered through glass wool and the filtrate collected. This preparation was employed in the experiments detailed in this paper unless otherwise stated.

The purification obtained by this method was variable within the range fivefold to tenfold. Table I shows the results of a typical purification of the enzyme. The activity recovered was low but this was not too important since only one fractionation was employed in the routine procedure. A second precipitation with $MgSO_4$ resulted in no further purification, but only in a decreased yield of enzyme.

Results

The enzyme preparation was found to be somewhat unstable in aqueous solution. There was a significant loss in activity when the preparation was dialyzed overnight at 0° C. Table II shows the effects of dialysis on the ability

TABLE II

Stability of lingcod muscle lipase

Preparation	μl CO_2 evolved/flask/hour in expts.:				
	1	2	3	4	5
Non-dialyzed	83	119	69	76	155
Dialyzed against 0.01 *M* $MgCl_2$	71	34		74	
Dialyzed against H_2O	42	14	47	45	75
Dialyzed against H_2O, $MgCl_2$ added to give concentration of 0.01 *M*			43	47	
Dialyzed against 0.01 *M* cysteine					75

NOTE: Substrate was 8.4×10^{-2} *M* triacetin. Dialysis overnight except expt. 2, which was for 72 hours.

of the enzyme to hydrolyze triacetin. The loss in activity with dialysis was reduced but not eliminated by dialyzing against 0.01 *M* $MgCl_2$. The addition of $MgCl_2$ to the enzyme preparation which had been dialyzed against water did not restore the loss in activity. The presence of cysteine during dialysis failed to prevent the destruction of the enzyme. The preparation was stable in the frozen state and could be kept at −20° C for 4 weeks without any loss of activity.

The lipolytic activity of the enzyme preparation was studied at a series of pH's from 6.34 to 7.84 using the manometric technique. The results (Fig. 1) show that the reaction rate increased with pH throughout the entire range. Studies at higher pH's could not be carried out by this method due to the limitations imposed by the bicarbonate–CO_2 buffer system. The higher pH range was studied using the titrimetric method. The results obtained are shown in Fig. 1. The pH of optimum activity was 8.2. At higher pH values the activity decreased sharply.

The hydrolysis of triacetin was studied at temperatures varying from 5° C to 45° C and the results are shown in Fig. 2. The rate of hydrolysis increased with temperature over the whole range employed. However, at 45° C and to a slight extent at 35° C a decrease in the reaction rate during the course of the 1 hour incubation was observed. No such loss in activity occurred at 5, 15, or

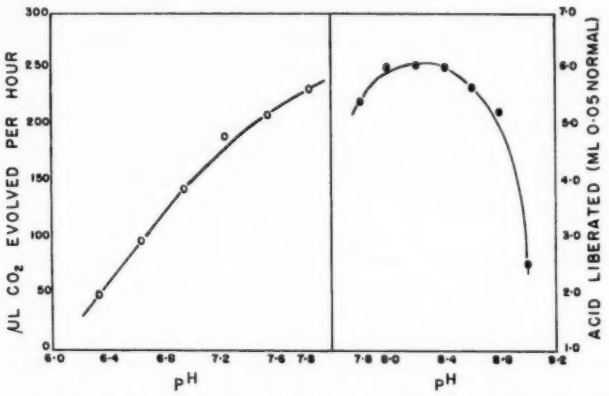

FIG. 1. Effect of pH on the rate of hydrolysis of triacetin by lingcod muscle lipase. Substrate concentration was 8.4×10^{-2} *M*, and 2.5×10^{-2} *M* for the manometric and titrimetric estimations respectively. The incubation for the latter method was 4 hours.

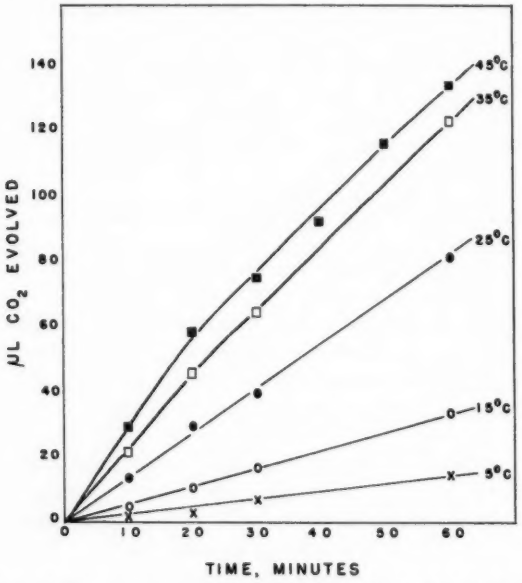

FIG. 2. Effect of temperature on the rate of hydrolysis of triacetin by lingcod muscle lipase. Substrate concentration was 8.4×10^{-2} *M*.

25° C. Moreover, the rate of hydrolysis at 45° C decreased when the amount hydrolyzed was less than that hydrolyzed after 1 hour at 25° C, at which time the reaction rate was still constant at the lower temperature. This indicates that the loss in activity at the higher temperatures was due to a destruction of the enzyme rather than to a shortage of substrate caused by the more rapid activity.

The effect of the lingcod muscle lipase on different substrates was studied (Table III). Both monoglycerides and triglycerides were attacked but methyl

TABLE III

Relative rates of hydrolysis of various esters by lingcod muscle lipase

Substrate	Conc.	Relative rate of hydrolysis (8.4×10^{-2} M triacetin = 500)
Triacetin	8.4×10^{-2} M	500
Tripropionin*	8.4×10^{-2} M	490
Tributyrin*	8.4×10^{-2} M	242
Trioctanoin*	8.4×10^{-2} M	2
Monoacetin	8.4×10^{-2} M	151
Monopropionin	8.4×10^{-2} M	233
Monobutyrin	8.4×10^{-2} M	295
Tween 20	5%	166
Methyl acetate	8.4×10^{-2} M	8
Ethyl acetate	8.4×10^{-2} M	13
Olive oil*	10%	3
Halibut liver oil*	10%	0
Lingcod liver oil*	10%	0

*Insoluble or not completely soluble in the concentrations employed and therefore added as a suspension.

acetate and ethyl acetate were hydrolyzed extremely slowly, if at all. The triglycerides containing longer chain fatty acids such as trioctanoin were not attacked. Olive oil, which is often used as a substrate in lipase studies, was not hydrolyzed by the fish muscle enzyme even when the oil was present as a very fine emulsion. Halibut liver oil and lingcod liver oil were likewise inactive. However, the enzyme was capable of hydrolyzing long chain fatty acids, as is shown by its action on Tween 20 which is the water-soluble monolaurate ester of a polyalkylene derivative of sorbitol. The results in Table III show that the rate of hydrolysis of the monoglycerides increased with the chain length of the fatty acid. The same increase in activity with chain length did not apply to the triglycerides. However, triacetin was soluble at the concentrations used whereas tripropionin and tributyrin were not completely soluble. In spite of this relative insolubility, tripropionin proved just as reactive as triacetin, while tributyrin showed half the activity. Triglycerides appeared

TABLE IV

The effect of various compounds on the hydrolysis of triacetin by lingcod muscle lipase

Compound	Conc., M	% control
p-Chloromercuribenzoate	1×10^{-3}	86
Potassium ferricyanide	2×10^{-3}	99
Sodium iodoacetate	2×10^{-3}	98
Sodium iodoacetate	1×10^{-2}	95
Potassium cyanide	1×10^{-5}	97
Potassium cyanide	1×10^{-3}	97
Potassium cyanide	1×10^{-2}	100
Sodium azide	1×10^{-3}	98
Sodium azide	1×10^{-2}	103

NOTE: Substrate concentration was 8.4×10^{-2} M.

to be more susceptible to attack than monoglycerides because triacetin was hydrolyzed more rapidly than monoacetin.

The effect of some common inhibitors of enzyme action on triacetin hydrolysis is shown in Table IV. Cyanide and azide in various concentrations had little or no effect on the activity. Of the sulphydryl reagents that were tested, *p*-chloromercuribenzoate and possibly iodacetate brought about inhibition of the lipase activity. However, the inhibition was never great even with the *p*-chloromercuribenzoate.

Discussion

The results presented here show that the lingcod muscle lipase was rather unstable in aqueous solution especially if no inorganic salts were present. This instability in solution is frequently encountered in lipase studies. Fiore and Nord (17) reported similar findings with *Fusarium lini* Bolley lipase, and they claimed that it was due to destruction of the enzyme by proteolysis. Singer and Hofstee (9), investigating wheat germ lipase, observed a loss in activity during overnight storage at 3° C. These workers, however, were of the opinion that the inactivation was due to oxidation of essential SH groups since the inactivation was reduced by the presence of glutathione. The destruction of lingcod muscle lipase does not appear to be due to SH oxidation because cysteine did not prevent the destruction of the enzyme. Moreover, sulphydryl poisons did not have a very marked effect on the activity. This is to be compared with the results obtained by Singer and Hofstee for their wheat germ lipase. Under similar conditions to those used in the present experiments, the wheat germ lipase was inhibited 87% by *p*-chloromercuribenzoate and 43% ferricyanide. The inhibition of the lingcod muscle enzyme by these compounds was 14% and 1% respectively.

The lingcod muscle lipase attacks both monoglycerides and triglycerides but has little effect on methyl acetate and ethyl acetate. This indicates that the enzyme is a lipase rather than an esterase acting on simple esters of low molecular weight acids. However, the enzyme is without action on trioctanoin and olive oil. This at first sight would indicate that the fish muscle enzyme does not hydrolyze molecules containing longer chain fatty acids. However, it was observed that lauric acid was split from the water-soluble Tween 20. In addition, the activity of the soluble monoglycerides increased with the chain length of the acid. The possibility therefore arises that it is not so much the chain length of the glyceride which is preventing attack but rather the insolubility of the compound.

The present investigation has shown that an enzyme exists in lingcod muscle which can hydrolyze lipid material. The possibility that the formation of free fatty acids during storage of fish fillets is due to enzymes in the muscle cannot therefore be ignored. At the temperatures employed for the storage of fish (−10° C to −20° C) the enzyme would of course have to act in firmly frozen material. Balls and Tucker (21) have shown that lipase enzymes can indeed act at low temperatures. They reported that an enzyme system from dried pig

pancreas was able to hydrolyze olive oil and tributyrin at temperatures as low as −25° C.

The results reported in this paper have concerned the action of a lipase which may possibly be acting on fish muscle to liberate free fatty acids. The phospholipase group of enzymes, however, cannot be ignored. The phospholipids are major components of the lipids in some species of fish. The lingcod belongs to this group and the release of fatty acids may be due to the action of phospholipases rather than lipases. An investigation is at present being conducted into the occurrence of phospholipases in fish muscle. The possibility that the hydrolysis is due to non-enzymic catalysis must, of course, always be borne in mind.

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LINGCOD MUSCLE NUCLEASE[1]

NEIL TOMLINSON

Abstract

A nuclease from muscle of lingcod (*Ophiodon elongatus*) has been purified by chromatography on diethylaminoethyl cellulose in the free-base form by stepwise elution with increasing concentrations of tris(hydroxymethyl)aminomethane. HCl, pH 7.0. The nuclease has been shown to hydrolyze ribonucleic acid, adenosine 3′-benzyl phosphate, thymidine 3′-*p*-nitrophenyl phosphate, and thymidine 5′-*p*-nitrophenyl phosphate. The latter compound was hydrolyzed at a very low rate. It did not hydrolyze adenosine 2′- or 5′- benzyl phosphate or certain nucleoside cyclic phosphates. The enzyme was inhibited by monoiodoacetate but not by heparin.

Introduction

The partial purification and some of the properties of an enzyme from the muscle of lingcod (*Ophiodon elongatus*) have been described (1). This enzyme degraded ribonucleic acid (RNA)* with the release of nucleoside 3′-phosphates. The present work describes the further purification of the enzyme by chromatography on DEAE, and the results of an investigation of the enzyme's substrate specificity.

As it has been found that the purified enzyme can hydrolyze certain simple esters of both ribo- and desoxyribomononucleotides, it will in future be called a nuclease, rather than a ribonuclease as in the previous publication (1).

Materials and Methods

The procedure for the preparation of muscle extracts of lingcod suitable for chromatographic treatment was as previously described (1), except that partial purification by isoelectric precipitation at pH 4.5 was omitted and the extract was heated 10 minutes at 58° C at pH 7.0 rather than at pH 9.0.

Protein and all light absorption measurements were made as previously described (1).

Temperature of incubation in all enzymatic assays was 25° C.

Nuclease and phosphodiesterase assays of fractions from chromatographic columns were performed by methods similar to those used previously (1), but modified to provide greater sensitivity. Thus, for assay of nuclease, the reaction mixture contained sodium succinate buffer pH 6.4, 0.025 *M*, RNA (95 μg RNA-P), and 0.5 ml of the fraction assayed in a total volume of 1 ml. Aliquots

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*ABBREVIATIONS: DEAE, diethylaminoethyl cellulose; DNA, desoxyribonucleic acid; RNA, ribonucleic acid; RNA-P, ribonucleic acid phosphorus; T3′-NPP, thymidine 3′-*p*-nitrophenyl phosphate; T5′-NPP, thymidine 5′-*p*-nitrophenyl phosphate; Tris, tris(hydroxymethyl)aminomethane; UTCA, uranyl acetate – trichloroacetic acid; 2′,3′-AMP, adenosine 2′,3′-cyclic phosphate; 2′,3′-UMP, uridine 2′,3′-cyclic phosphate.

(0.4 ml) of the reaction mixture were removed immediately after the addition of the enzyme and after a 2-hour incubation period. Each aliquot was at once mixed with 0.5 ml of MacFadyen's UTCA reagent (2) and 0.1 ml of water in a 75 mm × 10 mm test tube. The mixture was kept at room temperature for 30 minutes and was then centrifuged at 200 × *g* for 10 minutes. A 0.4-ml aliquot of the supernatant solution was carefully removed with a pipette and added to 0.8 ml of distilled water. The optical density of this solution at 260 mμ was determined with a suitable dilution of the UTCA reagent as reference blank. Reaction blanks with enzyme omitted were used. Results are reported simply as net increases in optical density at 260 mμ. Units of activity were not used in this work due to the impracticability of making corrections to compensate for the influence that different buffer concentrations of the various fractions assayed had on the enzyme's activity. Phosphodiesterase was assayed with bis (*p*-nitrophenyl)phosphate as substrate. The assay mixture contained sodium succinate buffer pH 6.4, 0.025 *M*, substrate 0.001 *M*, and 0.2 ml of the fraction assayed, in a total volume of 0.33 ml. After 4 hours' incubation, 0.8 ml of 0.1 *N* sodium hydroxide was added, and the *p*-nitrophenol released was determined as previously described (1).

Assays for enzymatic activity with *p*-nitrophenyl phosphate, T3′-NPP, or T5′-NPP as substrate were carried out as for phosphodiesterase activity. The time of incubation was reduced to 2 hours with certain exceptionally active fractions.

Assays for desoxyribonuclease activity were carried out by the method of Maver and Greco (3). The reaction mixture contained sodium succinate buffer pH 6.4, 0.025 *M*, DNA 0.5 mg/ml, and enzyme in a total volume of 0.4 ml. The incubation period was 4 hours.

Examination of the products of hydrolysis of RNA by the purified nuclease was made by means of two-dimensional paper chromatography and paper electrophoresis as before (1), but the nucleotides were eluted in 0.05 *M* sodium phosphate pH 7.0 rather than in 0.01 *N* HCl. The same procedure was used for quantitative measurement of nucleotides separated by chromatography or electrophoresis when the benzyl esters of the adenylic acid isomers or the cyclic ribomononucleotides (*vide infra*) were the substrates. For the latter the absorption data of Brown, Magrath, and Todd (4) were used.

The ability of the nuclease to hydrolyze 2′,3′-AMP and 2′,3′-UMP, and the influence of these two compounds on the hydrolysis of RNA by the enzyme, were investigated with a reaction mixture that contained either of the cyclic mononucleotides alone (0.006 *M*), or RNA (206 μg P/ml) with or without a cyclic mononucleotide (0.006 *M*), together with sodium succinate buffer pH 6.4, 0.025 *M*, chlortetracycline 5 p.p.m., and enzyme. Chlortetracycline does not inhibit either the nuclease or the phosphodiesterase at this concentration and was used to prevent bacterial growth in extended incubations. The total volume of the digestion mixture was 80 μl, and incubation was carried out in small, tightly stoppered test tubes. Controls without enzyme were used. Aliquots of 25 μl were removed at the beginning of the experiment and after 10 and 20 hours of incubation. When the mononucleotides alone were the substrate,

the aliquots removed were subjected to electrophoresis in sodium phosphate buffer pH 7.4, 0.2 *M* according to the method of Davis and Allen (5). When RNA with or without the mononucleotide was substrate, the aliquots were subjected to two-dimensional paper chromatography using isopropanol–water (70/30, v/v) with 0.35 ml ammonia solution (0.88 sp. gr.) for each liter of gas space in the tank (6) as the first solvent system, and with isobutyric acid 0.5 *N* and ammonium hydroxide pH 3.7 (7) as the second.

The ability of the enzyme to attack the benzyl esters of adenosine 2′-, 3′-, and 5′- phosphates was investigated in a manner identical to that used with the cyclic mononucleotides, including examination of the results by paper electrophoresis.

Column chromatography of proteins was carried out with jacketed columns refrigerated at 1° C and with the tubes in the fraction collector held in a bath of refrigerant at the same temperature.

DEAE type 20, capacity 0.85 meq/g was obtained from the Brown Co., Berlin, N. H. DNA was obtained from Worthington Biochemical Corporation, Freehold, N. J. Heparin (110 international units/mg) was obtained from the Connaught Medical Research Laboratories, Toronto, Ont. 2′,3′-AMP and 2′,3′-UMP, as the barium salts, were obtained from Schwarz Laboratories, Inc., Mount Vernon, N. Y. These two compounds were converted to their sodium salts by treatment with Dowex 50 (×8) in the sodium form before use. Adenosine 2′-, 3′-, and 5′-benzyl phosphates, T3′-NPP and T5′-NPP, and bis(*p*-nitrophenyl)phosphate were gifts from Drs. H. G. Khorana, J. G. Moffatt, W. E. Razzell, G. M. Tener, and A. Turner of the British Columbia Research Council, Vancouver, B.C. Disodium *p*-nitrophenyl phosphate was obtained from Sigma Chemical Co., St. Louis, Mo.

Results

The results of the chromatography of lingcod muscle extracts prepared as described above are shown in Figs. 1 and 2.

The DEAE used in the chromatogram of Fig. 1 was equilibrated with Tris.HCl, pH 6.9, 0.02 *M*, before the protein was added. Gradient elution was used to develop the chromatogram, the gradient being obtained by means of a single reservoir and 125-ml mixing flask. The mixing flask was filled initially with Tris.HCl, pH 6.9, 0.02 *M*, and the reservoir contained Tris.HCl, pH 5.8, 0.5 *M*. With this procedure, although three or four fractions containing nuclease were obtained free from phosphodiesterase activity (at least within the limits of sensitivity of the phosphodiesterase assay), separation of the nuclease from the phosphodiesterases was not good. The use of Tris.HCl, pH 5.8 or pH 7.0, 0.25 *M*, in the reservoir did not improve separation. In addition, it was observed that the buffered DEAE had a surprisingly low capacity for the protein contained in these preparations, for when 95 mg of protein, rather than 61 mg as in Fig. 1, was applied to a column of the same dimensions a much larger proportion of the total protein and a large part of the nuclease was not retained by the DEAE, indicating overloading. The

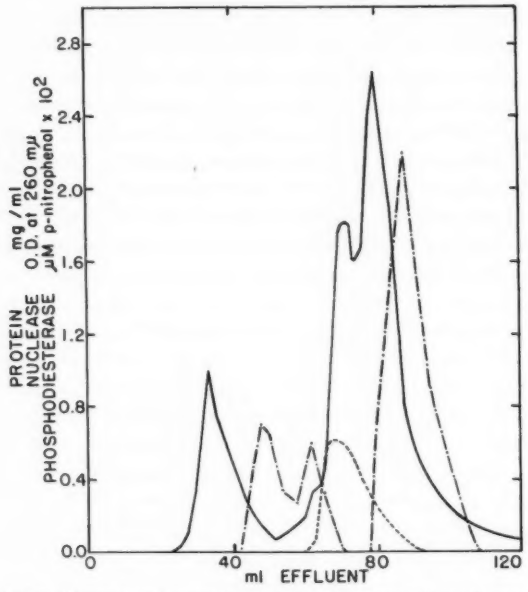

FIG. 1. Effluent diagram of protein preparation from lingcod muscle. Sixty-one milligrams of protein applied on 12 g DEAE previously equilibrated with Tris.HCl, 0.02 *M*, pH 6.9. Column 29 cm long × 18 mm diameter. Gradient elution with Tris.HCl (see text). Flow rate, 0.25 ml/min; fractions, 2 ml. First fraction collected after addition of protein. Hold-up volume *ca.* 38 ml. Protein ——. Nuclease----. Phosphodiesterase -·-·-.

capacity of DEAE in the free-base form for the protein was examined and was found to be very much greater than that of the buffered form. Mitz and Yanari (8) have reported the successful use of DEAE in the free-base form, with carbon dioxide solutions as eluent, in the chromatography of a number of proteins. In view of these facts, and the known moderate stability of the nuclease and phosphodiesterases under alkaline conditions (1), chromatography using DEAE in the free-base form and stepwise elution with increasing concentrations of Tris.HCl, pH 7.0, was investigated. Figure 2 presents the results. It is obvious that much better separation of the nuclease from the phosphodiesterases and greater purification of the nuclease with respect to protein were obtained. The three chromatograms show the influence of different degrees of loading of the DEAE with protein. In Fig. 2A the DEAE was very lightly loaded, in Fig. 2B it was slightly overloaded, and in Fig. 2C it was quite heavily overloaded. Moderate overloading can be used to advantage with the nuclease in order to obtain greater concentration of this activity in the effluent.

The nuclease activity accompanying the phosphodiesterase activity eluted with *M* Tris.HCl, and the small peaks of nuclease activity preceding the main nuclease peak in the overloaded column are regarded as artifactitious. Sober and Peterson (9) have pointed out the possibility of a condition such as this arising when stepwise elution is used. It is certain the nuclease and phospho-

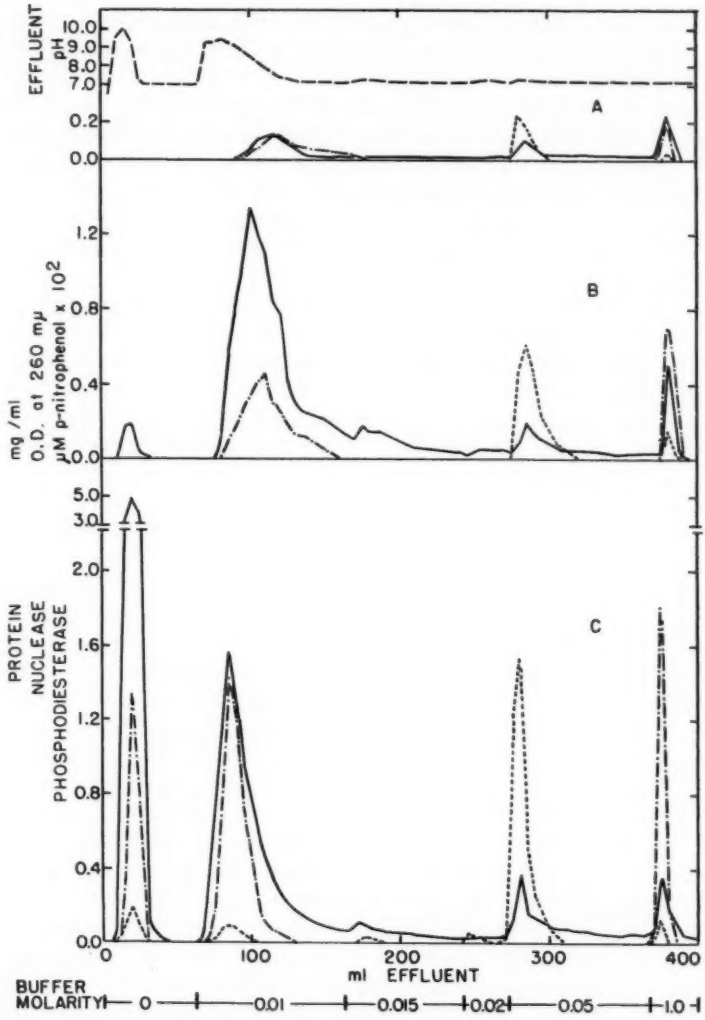

FIG. 2. Effluent diagrams of protein preparation from lingcod muscle. Each column contained 0.7 g DEAE in the free-base form. Protein applied: A, 14 mg; B, 75 mg; C, 120 mg. Column 2.5 cm long × 18 mm diameter. Stepwise elution, following distilled water wash, with Tris.HCl pH 7.0 at concentration indicated. Flow rate, 1 ml/min. First fraction collected during addition of protein. Hold-up volume *ca.* 6 ml. Fractions, 5 ml. Effluent pH for A – – –. Protein ——. Nuclease ----. Phosphodiesterase –·–·–.

diesterase eluted together are not identical, for a portion of each was obtained free from the other by chromatography on buffered DEAE (Fig. 1), and a preliminary experiment has indicated that the two can be separated on the free-base form by suitable intermediate steps in Tris.HCl concentration. It will be noted also that the quantity of nuclease activity eluted with the phospho-

diesterase by *M* Tris.HCl does not increase with increased protein added to the column as does the phosphodiesterase, presumably due to the displacement of the nuclease by more firmly bound material.

The effluent pH for the chromatogram of Fig. 2A is shown. For the chromatograms of Figs. 2B and 2C the changes in effluent pH are not shown, but were similar to those of Fig. 2A, reaching slightly higher values during addition of the protein and slightly lower values during the first passage of Tris.HCl through the column, with the peak value being displaced slightly to the left. The alkaline fractions were neutralized with cold *N* HCl immediately after they were collected.

The ability of the nuclease, following purification by either chromatographic procedure, to hydrolyze RNA, DNA, several simple esters of ribo- and desoxyribomononucleotides, bis(*p*-nitrophenyl)phosphate, and *p*-nitrophenyl phosphate has been examined.

The nuclease hydrolyzed RNA with the release of nucleoside 3′-phosphates, as previously reported (1), and hydrolyzed adenosine 3′-benzyl phosphate with the release of adenosine 3′-phosphate. However, using the assay conditions described above, with sufficient enzyme to provide over 90% hydrolysis of adenosine 3′-benzyl phosphate and hydrolysis of RNA to the extent that 70% of the RNA-P could be recovered as the nucleoside 3′-phosphates in 20 hours incubation, no hydrolysis whatever of adenosine 2′- or 5′-benzyl phosphates, of 2′,3′-AMP, or of 2′,3′-UMP could be detected. When either of the cyclic nucleotides was used together with RNA as substrate, 60 to 70% inhibition of the release of nucleoside 3′-phosphates was observed, with no detectable change occurring in the amount of the cyclic nucleotide. With adenosine 3′-benzyl phosphate as substrate no intermediate formation of the cyclic nucleotide could be detected, although this would have been readily found by means of the electrophoretic technique used if any appreciable amount had accumulated (5).

No hydrolysis of DNA, as measured by release of acid-soluble, ultraviolet-absorbing material (3), could be demonstrated, but the nuclease hydrolyzed T3′-NPP and T5′-NPP with the release of *p*-nitrophenol. The 5′-isomer was hydrolyzed at less than 2% of the rate of the 3′-isomer; 0.0005 μmole *p*-nitrophenol was released from the 5′-isomer against 0.031 μmole from the 3′- in 2 hours by 0.2 ml of a fraction from the main nuclease peak of Fig. 2C containing 138 μg protein/ml. It thus appears that the nuclease can hydrolyze phosphodiester linkages involving either ribose or desoxyribose. The very slow hydrolysis of T5′-NPP seems to be an exception to the apparent specificity of the enzyme for phosphodiester linkages in which the linkage not hydrolyzed must be to the 3′- position of ribose or desoxyribose. Possibly the *p*-nitro group influences the reaction in such a fashion as to modify the apparent specificity of the enzyme. A similar suggestion has been made by Schmidt (10) in connection with the activity of certain phosphomonoesterases. The possibility that the hydrolysis of T5′-NPP was due to a contaminating enzyme was considered. It cannot be due to contamination by the phosphodiesterases from which the nuclease appeared to be separated on the chromatogram. The

phosphodiesterase(s) preceding it from the column did not hydrolyze T5′-NPP, while the one following it did hydrolyze this compound, but was found not to be inhibited by 0.005 *M* monoiodoacetate, whereas the hydrolysis by the nuclease was inhibited almost completely. The fact that the nuclease purified by either chromatographic procedure hydrolyzed the same compounds, and that 0.005 *M* monoiodoacetate was found to inhibit the hydrolysis of each by more than 90%, suggested that one enzyme was probably concerned. No hydrolysis of either *p*-nitrophenyl phosphate or bis(*p*-nitrophenyl)phosphate could be detected. If the enzyme can hydrolyze either of these compounds it must be at a rate considerably less than 0.5% of that at which it hydrolyzed T3′-NPP.

The influence of heparin on the activity of the nuclease was investigated. Heparin is a powerful inhibitor of pancreatic ribonuclease (11, 12). At a concentration of 155 μg/ml, under assay conditions otherwise as described above, it produced no detectable inhibition of the lingcod nuclease with any of the substrates that the enzyme hydrolyzes.

Discussion

As has previously been pointed out (1) the lingcod muscle nuclease resembles Hilmoe and Heppel's (13) spleen nuclease fraction III (also called spleen phosphodiesterase (14)) in that they both hydrolyze RNA with the release of nucleoside 3′-phosphates, have similar pH optima and heat sensitivity, and do not require metallic ions for activation.

The present findings indicate several additional points of similarity. The spleen enzyme, like the lingcod nuclease, hydrolyzes ribomononucleoside 3′-alkyl phosphates but not the 2′- or 5′-esters (15), hydrolyzes T3′-NPP (16), and does not hydrolyze nucleoside 2′,3′-cyclic phosphates. Spleen nuclease fraction III hydrolyzed the nucleoside cyclic phosphates (15) but Hilmoe and Heppel subsequently freed it from this activity by means of an acetone fractionation procedure (16). However, in its apparent ability to hydrolyze T5′-NPP very slowly the lingcod nuclease differs from the spleen enzyme, with which no such activity has been detected. If the spleen enzyme does possess this activity, the rate of hydrolysis of T5′-NPP must be 0.01% or less of the rate for T3′-NPP (16).

The influence of monoiodoacetate and heparin on the activity of the lingcod enzyme is quite different from the influence these compounds have on the activity of the pancreatic ribonuclease. Monoiodoacetate is a very strong inhibitor of the lingcod enzyme with all the substrates yet known that it hydrolyzes, but the compound only inhibits the pancreatic enzyme in its attack on ribonucleic acid (17) and is without effect on its hydrolysis of simple esters of ribomononucleotides (5). Heparin inhibits all the activities of the pancreatic enzyme (11), but is without effect on the lingcod enzyme. It is interesting to speculate regarding the effect of heparin. It behaves as a competitive inhibitor of pancreatic ribonuclease (12, 18), and consequently its failure to inhibit the

lingcod nuclease suggests that the two enzymes have quite different active sites.

Acknowledgments

My sincere thanks are due to Drs. H. G. Khorana, J. G. Moffatt, W. E. Razzell, G. M. Tener, and A. Turner, British Columbia Research Council, for their very generous gifts of various nucleotide esters used in this work.

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SYNTHESIS OF SATURATED AND UNSATURATED L-α-LECITHINS

ACYLATION OF THE CADMIUM CHLORIDE COMPOUND OF L-α-GLYCERYLPHOSPHORYLCHOLINE[1]

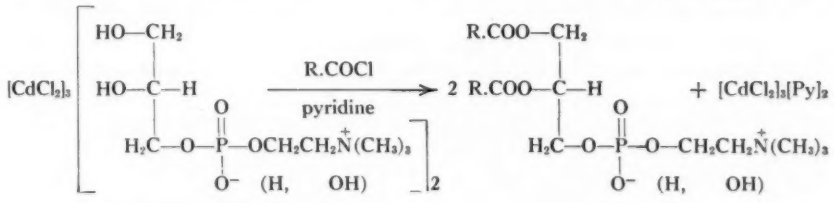
ERICH BAER AND DMYTRO BUCHNEA

Abstract

A facile procedure for the preparation of saturated and unsaturated L-α-lecithins from L-α-glycerylphosphorylcholine (GPC) is reported. The lecithins are obtained by acylating L-α-GPC, in the form of its cadmium chloride addition compound, with fatty acid chloride and pyridine at low temperature (0°–25°). The acylation proceeds rapidly and yields *optically pure* L-α-lecithins. The preparation of the distearoyl-, dimyristoyl-, and dioleoyl L-α-lecithins by this procedure is described.

The deacylation of lecithins with mercuric chloride or sodium hydroxide was investigated. Both procedures give a partially racemized L-α-GPC. This was established by the finding that the optical activity of the lecithins resulting from reacylating the L-α-GPC preparations in the form of their cadmium chloride compounds was considerably lower than that of the corresponding authentic lecithins.

In 1948, Baer and Kates (1) reported the synthesis of L-α-glycerylphosphorylcholine (L-α-GPC). Attempts to obtain lecithins by acylating the L-α-GPC with a fatty acid chloride and pyridine under a variety of experimental conditions were unsuccessful. The reaction mixtures consisted mainly of lysolecithins (2). As the acylation of the related but nitrogen-free L-α-glycerylphosphoryl ethylene chlorohydrin goes readily to completion under similar conditions (3), it was obvious that the presence of the quaternary ammonium group made it difficult for the acylation to proceed beyond the lysolecithin stage. The lecithins finally became accessible from GPC when one of the present authors (E.B.) found that its acylation can be carried to completion also if the amorphous cadmium chloride compound of L-α-GPC is used. The acylation of this compound, in which the cadmium chloride presumably forms a complex with the quaternary ammonium base, proceeds rapidly at low temperature (0°) and is essentially finished in approximately 2 hours (see Reaction Scheme). The L-α-lecithins are obtained in fairly good yields, and



L-α-Glycerylphosphorylcholine cadmium chloride compound

L-α-Lecithin

R = $-(CH_2)_{16}CH_3$, $-(CH_2)_{12}CH_3$, $-(CH_2)_7CH{=}CH(CH_2)_7CH_3$

REACTION SCHEME

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Contribution from the Subdepartment of Synthetic Chemistry in Relation to Medical Research, Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario.

are both optically and chromatographically pure. However, since the preparation of L-α-GPC by the method of Baer and Kates (1) was rather time consuming, and in the meantime other satisfactory methods for the synthesis of saturated (2, 4) and unsaturated L-α-lecithins (3) had been developed in this laboratory no detailed report of the synthesis of lecithins by acylation of the cadmium chloride compound of L-α-GPC was made. It was mentioned, however, in a discussion following a lecture given by one of the authors at a Symposium on the Chemistry and Physiology of Phospholipids at the University of Western Ontario (5).

We were therefore much interested in reports by Tattrie and McArthur (6) and Dawson (7) describing the facile preparation of L-α-GPC from purified egg-yolk lecithin by deacylation with mercuric chloride (6) or sodium hydroxide (7), respectively, and especially in a somewhat later report by Tattrie and McArthur describing the reacylation of L-α-GPC to L-α-(dipalmitoyl)lecithin (8). The lecithin was prepared by treating L-α-GPC, obtained from egg-yolk lecithin, with palmitoyl chloride at 37° for 72 hours, separating the mixture of acylation products on a silicic acid column, and reprecipitating the best lecithin fraction from chloroform with acetone. The L-α-(dipalmitoyl) lecithin, which was obtained in a yield of 30% (8), analyzed correctly for nitrogen and phosphorus. Its specific rotation of +5.5° was, however, considerably lower (17%) than that of authentic L-α-(dipalmitoyl)lecithin (+6.6°) (2), suggesting that the product was partially racemized. Whether the racemization had taken place during the deacylation of the lecithin or the reacylation of L-α-GPC to lecithin was not immediately obvious. Since Tattrie and McArthur reported for the L-α-GPC preparation from egg-yolk lecithin a specific rotation ($[\alpha]_D$ −2.89°) which is identical with that reported (1) for synthetic L-α-GPC ($[\alpha]_D$ −2.85°), it would appear unlikely that any racemization occurs during the deacylation of the lecithin. However, as it is difficult to measure the fairly small optical activity of L-α-GPC with an accuracy that would allow one to dismiss this possibility entirely, it was felt that the deacylation process should be checked for racemization. For this purpose an optically pure and well-defined synthetic L-α-(distearoyl)lecithin ($[\alpha]_D$ +6.2°) was deacylated with mercuric chloride following Tattrie and McArthur's description (6), and the L-α-GPC was isolated as cadmium chloride compound. As the specific rotation of this compound and that of L-α-GPC itself are rather small for an accurate check of their optical purity, the cadmium chloride compound was immediately reacylated with stearoyl chloride and pyridine (experiment 4). This procedure, as shown by experiments 1, 2, and 3, yields optically pure L-α-lecithins from optically pure L-α-GPC. The L-α-(distearoyl)lecithin thus obtained possessed, however, a specific rotation of +5.0°, a value about 20% lower than that of authentic L-α-(distearoyl)lecithin (2). Thus obviously the deacylation of lecithins with mercuric chloride under the conditions specified by Tattrie and McArthur produces a partially racemized L-α-GPC. That this most likely applies also to the deacylation of lecithins with sodium hydroxide is suggested by the specific rotation of −2.5° reported by Dawson (7) for L-α-GPC prepared by this method. Further evidence confirming the formation of a

partially racemized L-α-GPC under these conditions was obtained by preparing L-α-GPC from purified egg-yolk lecithin (9) by these two methods, and reacylating the cadmium chloride compounds of both preparations of L-α-GPC with stearoyl chloride and pyridine (experiments 5 and 6). The L-α-(distearoyl) lecithins thus obtained possessed again lower specific rotations than the authentic compounds by 11 and 15%, respectively.

Having thus established beyond doubt that the deacylation of lecithins with mercuric chloride or sodium hydroxide yields preparations of L-α-GPC that are racemized to varying degrees, it is obvious that L-α-GPC obtained by these methods is not a suitable starting material for the synthesis of *optically-pure* L-α-lecithins. Whether the recently reported procedure by Urakami and Okura (10) for the reductive deacylation of lecithins with lithium aluminum hydride yields L-α-GPC that will be suitable as starting material for the synthesis of optically pure lecithins has yet to be established.

The elegant method of Uziel and Hanahan (11) for the preparation of L-α-GPC from L-α-(dipalmitoleyl)lecithin involving the stepwise enzymatic removal of the fatty acid substituents unfortunately does not appear to lend itself to the preparation of larger amounts of L-α-GPC. However, the ease with which the saturated and unsaturated L-α-lecithins are obtained by acylation of the cadmium chloride compound of L-α-GPC made it highly desirable that the latter should be made more readily available by synthesis than it is now (1). Attempts to simplify its synthesis were successful and the shorter procedure will be reported elsewhere. In the experimental part of this paper we describe the synthesis of the distearoyl-, dimyristoyl-, and dioleoyl-L-α-lecithins. These are identical in every respect with the corresponding lecithins prepared by our other methods (2, 3, 4, 12). The synthesis of lecithins from L-α-GPC containing either shorter or more highly unsaturated fatty acid substituents is in progress in this laboratory. The possibility of obtaining "mixed-acid" L-α-lecithins by acylation of the cadmium chloride compounds of lysolecithins is also being investigated.

Experimental

(1) L-α-(*Distearoyl*)*lecithin from Synthetic* L-α-*GPC*

In a dry three-necked 500-ml flask equipped with an oil-sealed stirrer, calcium chloride tube, and dropping funnel were placed 100 ml of glass beads (5 mm in diameter) and 11.0 g (0.01 mole) of the amorphous cadmium chloride addition compound of synthetic L-α-glycerylphosphorylcholine (1). The flask was immersed in an ice-water bath, and to the rapidly stirred mixture was added in a thin stream a solution of 60.6 g (0.2 mole) of freshly prepared stearoyl chloride (13) in 60 ml of anhydrous and ethanol-free chloroform,[2] followed by a solution of 11 ml (0.14 mole) of anhydrous pyridine[3] in 100 ml of dry chloroform. After 30 minutes the temperature of the bath was raised to 25°, and the

[2]Immediately before use the chloroform (U.S.P.) was dried and freed of ethanol by distillation over phosphorus pentoxide.

[3]The dry pyridine was prepared by boiling it under reflux with barium oxide and distilling it under anhydrous conditions.

stirring was continued for 2 hours at this temperature. The reaction mixture was then poured through a Buchner funnel (without filter), the glass beads were rinsed with three 50-ml portions of chloroform, and the combined filtrates were cleared by centrifugation. The solvent was removed by distillation under reduced pressure, and the residue was freed of most of the pyridine by keeping it for several hours in a vacuum of 0.1 mm at a bath temperature of 30°–35°(*). The product then was suspended, with stirring, in 500 ml of anhydrous acetone[4] for 10 minutes and the mixture was separated by centrifugation. The precipitate was treated in the same manner successively with two 100-ml portions of anhydrous acetone and two 100-ml portions of anhydrous ether. The remaining solid material was dried under reduced pressure and freed of last traces of cadmium chloride and pyridine hydrochloride, by dissolving it in 200 ml of a mixture of chloroform, methanol, and water (5:4:1, v/v/v), and passing the solution through a column of 120 cm in length and 2.5 cm in diameter containing a mixture of equal volumes of Amberlites IR-45 and IRC-50. The column was washed with 500 ml of the chloroform, methanol, and water mixture, the combined effluents were evaporated to dryness under reduced pressure from a bath at 40°–45°, and the residue was dried in a vacuum of 0.1 mm at 45°. The crude lecithin was purified by precipitation from a solution in 50 ml of chloroform with 150 ml of acetone, separation of the mixture by centrifugation, and recrystallization of the precipitate, 7.7 g (47.6% of theory), from chloroform and ether as described by one of the authors (12). The recovered L-α-(distearoyl)lecithin weighed 6.2 g (38.4% of the theoretical yield). $[\alpha]_D^{29}$ +6.2° in chloroform–methanol (1:1, v/v), *c* 10, m.p. 231°–232°.[5] Reported for L-α-(distearoyl)lecithin (2, 4): $[\alpha]_D$ +6.1° in chloroform (*c* 10), m.p. 230°–232°. *Anal. Calc.* for $C_{44}H_{90}O_9NP$ (808.2): N, 1.73; P, 3.83. Found: N, 1.80; P, 3.90.

(2) L-α-*(Dimyristoyl)lecithin from Synthetic* L-α-*GPC*

The acylation of 11.0 g (0.01 mole) of the amorphous cadmium chloride compound of synthetic L-α-GPC (1) with 40.0 g (0.16 mole) of myristoyl chloride (13) and 15.0 ml (0.18 mole) of anhydrous pyridine was carried out as described above for distearoyl lecithin, following the description to the point indicated by an asterisk. The material thus obtained was taken up in 200 ml of anhydrous ether, the undissolved material, mainly pyridine hydrochloride, was removed by centrifugation, washed with anhydrous ether, and the combined ether solutions were evaporated to dryness under reduced pressure from a bath at 25° to 35°. The residue was taken up in 250 ml of anhydrous acetone, the solution was cleared by centrifugation and kept overnight at −10°. The mixture was separated by centrifugation at −10° (refrigerated centrifuge), and the precipitate of lecithin was triturated successively with three 100-ml portions of ice-cold anhydrous acetone, the mixtures being separated by centrifugation in the cold (−10°). The lecithin then was dried, dissolved in 200 ml of a mixture of chloroform, methanol, and water (5:4:1, v/v/v), and the

[4]The acetone was dried by shaking it with anhydrous potassium carbonate for 24 hours, and distillating it under anhydrous conditions.

[5]All melting points were determined in capillary tubes, using an electrically heated bath of *n*-butyl phthalate, and short-stem thermometers with a range of 50 degrees.

solution was passed through a column of 120 cm in length and 2.5 cm in diameter containing a mixture of equal volumes of Amberlites IRC-50 and IR-45. The column was washed with 500 ml of the same solvent mixture, the combined eluates were evaporated under reduced pressure from a bath at 35°–40°, and the residue was dried thoroughly in a vacuum of 0.1 mm. For further purification, the lecithin was triturated with 100 ml of anhydrous acetone, the colorless product was dried *in vacuo*, and recrystallized from chloroform and ether as described by Baer (12). The L-α-(dimyristoyl)lecithin (7.1 g, 51.0% of theory) was analytically pure; m.p. 236°–237°, $[\alpha]_D^{22}$ +7.0° in chloroform–methanol (1:1, v/v), *c* 10. Reported for L-α-(dimyristoyl)lecithin (2, 4): m.p. 236°–237°, $[\alpha]_D^{24}$ + 7.0° in chloroform–methanol (1:1, v/v). *Anal. Calc.* for $C_{36}H_{74}O_9NP$ (695.6): N, 2.01: P, 4.46. Found: N, 2.00; P, 4.43.

(3) L-α-(*Dioleoyl*)*lecithin from Synthetic* L-α-*GPC*

The acylation of 11.0 g (0.01 mole) of the amorphous cadmium chloride compound of synthetic L-α-GPC with 60.2 g (0.20 mole) of oleoyl chloride (13) and 11 ml (0.14 mole) of anhydrous pyridine, and the isolation of the acylation product were carried out as described for L-α-(distearoyl)lecithin (experiment 1) following the description to the asterisk. The material thus obtained was dissolved in 200 ml of anhydrous ether, the solution was cleared by centrifugation, and the solvent was removed under reduced pressure.[6] The residue then was dissolved in 600 ml of anhydrous acetone, and the solution was placed in a bath of solid carbon dioxide and acetone at −85° for 2 hours. The lecithin was isolated at as low a temperature as possible using a refrigerated centrifuge, and was precipitated once more from 300 ml of acetone as described above. The precipitate was dried by bringing it slowly to room temperature in a desiccator while gradually lowering the pressure. This material, weighing 15.5 g, was dissolved in 200 ml of a mixture of chloroform, methanol, and water (5:4:1; v/v/v), and the solution was passed through a column, 120 cm in length and 2.5 cm in diameter, containing a mixture of equal volumes of Amberlites IR-45 and IRC-50. The column was washed with 400 ml of the solvent mixture, the combined effluents were freed of solvents by distillation under reduced pressure from a bath at 35°–40°, and the residue was dried in a vacuum of 0.1 mm at 40°. It then was dissolved in 250 ml of chloroform, and the solution was passed through a column 60 cm in length and 4.5 cm in width containing 400 g of silicic acid[7] (Merck, Reagent Grade). The column was washed with chloroform until the effluent was free from solute (mainly oleic acid), and the lecithin was recovered by passing through the column approximately 1.5 liters of a mixture of methanol and chloroform (1:2, v/v), removing the solvents by distillation under reduced pressure from a bath at 35°–40°, and drying of the residue in a vacuum of 0.1 mm. The slightly amber-colored[8] L-α-(dioleoyl)-lecithin, a wax-like material, weighed 8.8 g (54.7% of theory) and analyzed

[6]This and all following vacuum distillations during the preparation of the unsaturated lecithin were carried out in an atmosphere of nitrogen; the nitrogen being introduced through a capillary.

[7]The silicic acid was sifted through a sieve of 150 meshes per linear inch to remove particles smaller than 100 microns.

[8]Introduced by the Amberlites.

correctly for nitrogen and phosphorus: Calc.: N, 1.74; P, 3.85. Found: N, 1.78; P, 3.81. $[\alpha]_D$ +6.0, in chloroform–methanol (1:1, v/v). To remove the color, the lecithin was dissolved in 35 ml of anhydrous, peroxide-free ether, and reprecipitated by the slow addition of 70 ml of anhydrous acetone. The mixture was separated by centrifugation, and the precipitate was dried in a vacuum of 0.1 mm at room temperature over sodium hydroxide pellets and phosphorus pentoxide. The almost colorless L-α-(dioleoyl)lecithin weighed 8.2 g (93% of recovery). Over-all yield 51%. $[\alpha]_D$ +6.1° in chloroform–methanol (1:1, v/v), *c* 10. Reported (3) $[\alpha]_D$ +6.2°. *Anal. Calc.* for $C_{44}H_{86}O_9NP$ (804.2): N, 1.74; P, 3.85. Found: N, 1.78; P, 3.82.

When chromatographed on paper impregnated with silicic acid (14), the dimyristoyl-, distearoyl-, and dioleoyl-L-α-lecithins prepared as described above produced single spots suggesting that they are pure substances.

L-α-(Distearoyl)lecithin

(4) *Prepared from* L-α-*GPC Obtained by Deacylation of Synthetic* L-α-*(Distearoyl)lecithin with Mercuric Chloride*

L-α-(Distearoyl)lecithin (2, 4, 12) ($[\alpha]_D$ +6.2°) was hydrolyzed with mercuric chloride by Tattrie and McArthur's procedure (6), and the resulting L-α-GPC was converted to the amorphous cadmium chloride compound (1). Reacylation of 0.65 g of the cadmium chloride compound with stearoyl chloride and pyridine, and the isolation and purification of the acylation product as described above (experiment 1) gave 0.40 g (41.9% of theory) of L-α-(distearoyl)lecithin $[\alpha]_D$ +5.0° in chloroform–methanol, *c* 10. Rotation of authentic material (2) $[\alpha]_D$ +6.1° in chloroform–methanol. *Anal. Calc.* for $C_{44}H_{90}O_9NP$ (808.2): N, 1.73; P, 3.83. Found: N, 1.74; P, 3.82.

(5) *Prepared from* L-α-*GPC Obtained by Deacylation of Egg-Yolk Lecithin with Mercuric Chloride*

Purified egg-yolk lecithin, prepared by the method of Hanahan *et al.* (9, 11) was deacylated by hydrolysis with mercuric chloride as described by Tattrie and McArthur (6), and the resulting L-α-GPC was converted to the amorphous cadmium chloride compound (1). Reacylation of 3.1 g of the cadmium chloride addition compound with stearoyl chloride and pyridine, and the isolation and purification of the acylation product as described above (see experiment 1), gave 2.2 g (48.3% of theory) of L-α-(distearoyl)lecithin. $[\alpha]_D$ +5.5° in chloroform–methanol (1:1, v/v), *c* 10. Authentic L-α-(distearoyl) lecithin (2) $[\alpha]_D$ +6.1° in chloroform–methanol. *Anal. Calc.* for $C_{41}H_{90}O_9NP$ (808.2); N, 1.73; P, 3.83. Found: N, 1.72; P, 3.82.

(6) *Prepared from* L-α-*GPC Obtained by Deacylation of Egg-Yolk Lecithin with Sodium Hydroxide*

Purified egg-yolk lecithin, prepared by the method of Hanahan *et al.* (9, 11), was deacylated by saponification with sodium hydroxide as described by Dawson (7), and the resulting L-α-GPC was converted to the amorphous cadmium chloride compound (1). The acylation of 3.4 g of this compound with stearoyl chloride and pyridine, the isolation and purification of the acylation

product as described above (see experiment 1), gave 2.2 g (44% of theory) of L-α-(distearoyl)lecithin. $[\alpha]_D$ +5.2° in chloroform–methanol (1:1, v/v), *c* 10. Reported (2) $[\alpha]_D$ +6.1°. *Anal. Calc.* for $C_{44}H_{90}O_9NP$ (808.2): N, 1.73; P, 3.83. Found: N, 1.77; P, 3.86.

Acknowledgment

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LINGCOD MUSCLE PHOSPHORIBOISOMERASE AND RIBULOSE 5′-PHOSPHATE 3′-EPIMERASE[1,*]

H. L. A. TARR

Abstract

Comparatively pure phosphoriboisomerase and ribulose 5′-phosphate 3′-epimerase enzyme preparations were obtained from lingcod muscle by a simple procedure involving water extraction, saturation of the extract with ammonium sulphate, dialysis, brief heating to 55° C, lyophilization of the solution, and final separation by ion exchange chromatography, using diethylaminoethyl cellulose columns. Both enzymes have broad pH optima above pH 7.0, but are rapidly inactivated below this value. The following equilibria were established and compared with those obtained by other investigators: R5P ⇄ RU5P, 1.35:1.0; RU5P ⇄ XU5P, 1:1.5 and R5P ⇄ RU5P ⇄ XU5P, 1:0.58:0.66. The ketopentulose phosphate resulting from the action of phosphoriboisomerase on D-ribose 5-phosphate was isolated and identified as D-ribulose 5-phosphate. Both D-ribulose and D-xylulose were demonstrated after subjecting a product of epimerase action to hydrolysis by acid phosphatase and ion exchange chromatography.

Previous investigations have been concerned with the nucleoside hydrolase and phosphorylase enzymes of lingcod (*Ophiodon elongatus*) muscle, and with a crude phosphoriboisomerase enzyme preparation from this source by the action of which deoxyribose 1,5-diphosphate was prepared for the first time (1).

The importance of pentose phosphate esters in biochemical processes has become increasingly apparent in recent years. Thus, oxidative decarboxylation of glucose 6-phosphate via the so-called hexose monophosphate shunt pathway leads to formation of RU5P (2). Two enzymes are required to transform this compound into substrates for transketolase. These are epimerase, which catalyzes formation of XU5P, the donor, and PRI, which catalyzes formation of R5P, the acceptor aldehyde. The present paper describes the isolation, partial purification, and some of the properties of these enzymes as obtained from lingcod muscle.

Materials

o-Nitrophenylhydrazine used in preparation of ribulose *o*-nitrophenylhydrazone was made from *o*-nitroaniline (3). D-Ribulose *o*-nitrophenylhydrazone was prepared by the method of Glattharr and Reichstein (4) and recrystallized from absolute methanol. The melting point and specific rotation agreed well with those obtained by other investigators: $[\alpha]_D^{21}$ +51.8° in methanol (*c*, 1.35); m.p. 165.5–166° C (tube); 163–164° C (block) (3, 4). D-Xylulose *p*-bromophenylhydrazone was prepared as described by Schmidt and Treiber (5). This

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*The following abbreviations are used: R5P, D-ribose 5-phosphate; RU5P, D-ribulose 5-phosphate; XU5P, D-xylulose 5-phosphate; PRI, phosphoriboisomerase; EPIM, ribulose 5′-phosphate 3′-epimerase; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethylcellulose; XU, xylulose; RU, ribulose.

compound (m.p. 128–128.5° C) exhibited the pronounced mutarotation noted by the above workers: $[\alpha]_D^{21}$ +19.9°–20.6° 15–20 minutes after dissolving in anhydrous pyridine; (*c*, 2.094) and $[\alpha]_D^{2}$ −34.2° to −35.6° after 7–8 days at this temperature. These compounds were used directly as standards in the cysteine–carbazole reaction (6), and were decomposed as described by Schmidt and Treiber (5) to obtain the free pentuloses for chromatographic standards.

Barium R5P was prepared from chromatographically pure 5′-adenylic acid (Schwarz) and was dried in high vacuum over P_2O_5 (7). This compound was practically free from orthophosphate, possessed about the theoretical organic phosphorus and pentose content (based on mol. wt. 365.5), and gave the specific rotation characteristic of R5P of high purity (2); $[\alpha]_D^{23}$ +23.2° in 0.2 *N* HCl (*c*, 1.909) based on the free acid, mol. wt. 230). The RU5P content was 2–3% as determined by the cysteine–carbazole reaction and the presence of alkali-labile pentose. The enzymic preparation of RU5P is described later.

DEAE cellulose Type 20 was obtained from the Brown Co., Berlin, New Hampshire. Dowex 1×10 and Dowex 50×8 (200–400 mesh, analytical grades) were obtained from the California Foundation for Biochemical Research, and Amberlite IR 45 (analytical grade) from the Fisher Scientific Co.

Methods

Aldopentose and aldopentose phosphates were determined by the orcinol procedure (8) with a 40-minute heating period and arabinose as standard. The optical density at 670 mμ with pentulose phosphates in this method was multiplied by 1.76 in order to determine the concentration present, since these compounds give about 57% of the value of an identical amount of arabinose on the basis of the pentulose content (9, 10). With this method the average ratios of absorbancy at 540/670 mμ were: D(−)ribose, 0.21; D(−) and L(+)arabinose, 0.19; D(−)ribulose, 0.85; D(−)xylulose, 0.49; and D-RU5P, 0.33.

Alkali-labile pentulose was determined by adjusting the sample to 1.0 *N* with 2 *N* NaOH, holding 20 minutes at 25°, and then carrying out the orcinol reaction (11). Under these conditions pentuloses appear to be completely inactivated while R5P is very resistant. Tests indicated that approximately 4% of R5P is inactivated during this treatment, and this was taken into consideration when the method was used.

Pentuloses and pentulose phosphates were also determined directly by the cysteine–carbazole reaction (6) after 15 minutes for ribulose and 120 minutes at 30° for xylulose. The proportion of ribulose and xylulose in mixtures containing between 10 and 30 μg of the pentuloses was determined by calculating the ratio of the optical density at 540 mμ in the cysteine–carbazole reaction after 15 minutes and after 120 minutes. In this test the ratio with ribulose alone was 1.04 and with xylulose alone 0.55. This method required prior hydrolysis of the pentulose phosphates with acid phosphatase (see below) but proved more sensitive than that of Ashwell and Hickman (12), in which the proportion of pentulose phosphates is determined directly using a modified cysteine–carbazole reaction. Pentulose phosphates were determined directly using the cysteine–

carbazole reaction with a 2-hour heating period at 37°. Under these conditions experience showed that the amount of pentulose found multiplied by the factor 1.85 gave the concentration of pentulose in pentulose phosphate.

Protein was determined by the quantitative biuret method (13) or by that of Lowry *et al.* (14), the latter proving exceedingly useful where only small amounts of protein were measured. The method of Lowry and Lopez (15) with the modification of Bruemmer and O'Dell (16), and also that of Gomori (17) were used to determine orthophosphate, after appropriate hydrolysis procedures where necessary.

Acid phosphatase was prepared from "Polidase" (Schwarz), using a recent modification (9) of the original procedure of Hochster (18). Its activity (226 μmoles of R5P hydrolyzed by 1.0 ml in 30 minutes at 37°) was similar to that reported by the above investigators.

Barium ions were removed from Ba R5P and Ba RU5P by passage through small columns of Dowex 50 H^+ resin, adjusting the effluent and washings to pH 7–7.5 with NaOH or KOH as required, and standardizing the solutions by the appropriate methods.

5-Phosphoribonic acid was prepared by oxidation of Ba R5P with alkaline periodate (19) to yield the tricalcium salt $(C_5H_8O_9P)_2Ca_3$ (20) (P calc. 10.23; found 9.77).

All enzyme reactions were carried out at 37° C. Chromatographic separation of the R5P and RU5P was carried out using 16-hour descending development on Whatman No. 1 paper with *n*-propanol:28% NH_4OH:H_2O (6:3:1). Separation of the free pentuloses was effected by 16-hour ascending development, using 90% phenol as solvent in presence of KCN crystals. The usual spray reagents were employed (9, 18, 21). The enzyme reactions were carried out in 160×15 mm tubes, the appropriate colorimetric reaction being carried out in the same tube.

Experimental

Preparation of Enzymes

In this work one unit of enzyme is arbitrarily defined as that amount which caused turnover of 1 micromole of substrate in 1 minute at 37° C where conditions were such that the reaction was approximately linear with respect to time (22). The specific activity is recorded as units per milligram of protein.

Satisfactory preparations were obtained from fresh or frozen lingcod, and all procedures were carried out at 0 – 3° C. In a typical case 1.5 kg of the muscle of a fish which had been frozen and stored about 3 months at −20° C was thawed until it could be cut readily, and was then blended for about 1 minute with 3 liters of water and sufficient 1 *N* HCl to adjust the suspension to pH 6.0. The suspension was centrifuged 15 minutes at 14,000 *g*. The supernatant liquid was filtered through glass wool to yield 1460 ml of fairly clear liquid. This was saturated with $(NH_4)_2SO_4$ (2.46 kg) at pH 7.0, about 35 ml of 2 *N* NaOH being required to adjust the reaction. The precipitated protein was collected by filtering for 16 hours at 0° C through Whatman No. 1 paper. The moist filter cake (about 250 g) was dialyzed 2 hours against running tap

water (7° C) and then for 24 hours against several changes of demineralized water to yield 870 ml of crude enzyme preparation (16.8 mg of protein/ml) (Table I).

TABLE I

Purification of PRI

Fraction	Ml	Mg protein per ml	Total protein, mg	Specific activity	Total units
Saturated $(NH_4)_2SO_4$	870	16.8	14,700	0.175	2,565
Heated to 55° C and lyophilized	179	39.4	7,050	0.284	2,000
Eluate from DEAE column	4,350	0.015	65	20.4	1,350

Crude enzyme (170 ml), in a stainless steel tube immersed in a 65° C water bath, was stirred rapidly until the temperature attained 55° C, and was then promptly chilled to 0° C. The coagulated protein was removed by centrifuging 5 minutes at 10,000 *g*. The clear supernatant liquid was lyophilized and the residue suspended in water, insoluble protein being removed by centrifuging. The pH, which was about 5.5, was cautiously adjusted to pH 7.0 with a little 1 *N* NaOH to yield 35 ml of a solution containing 39.4 mg protein/ml.

A jacketed column containing 13 g dry weight of DEAE (23) (34×1.8 (diam.) cm) was conditioned with 0.02 *M* Tris–HCl buffer, pH 7.0, and 6 ml (236 mg protein) of the lyophilized enzyme preparation in 0.02 *M* Tris was run onto the column, which was maintained at 1° C. The column was eluted at 1.3 ml/minute under 5 lb/sq. in. pressure, using a gradient in which 0.25 *M* Tris–HCl buffer was run into 250 ml of 0.02 *M* Tris–HCl, 5 ml fractions being collected. The chilled fractions were tested for protein and for PRI and EPIM activity. These eluates were conveniently buffered at pH 7–7.3 so could be assayed directly.

The position of PRI was determined by incubating 0.1 ml of the column eluates directly with 0.02 ml of a 10 μmole/ml solution of Na R5P, and determining the RU concentration by the 2-hour cysteine–carbazole procedure. The position of EPIM was determined by three different procedures as follows: (1) 0.04 ml of eluate was incubated with 0.02 ml of Na RU5P solution (0.2 μmole) at pH about 7.2 for 30 minutes at 37° C. The solutions were promptly chilled to 0°, 0.01 ml of 0.004% methyl red indicator, 0.01 ml of 0.01 *M* $MgCl_2$, and sufficient 0.1 *M* CH_3COOH to adjust the pH to about 5.0 (0.01–0.015 ml) were then added. After addition of 0.005 ml of acid phosphatase to each, the tubes were again incubated as above and the proportions of xylulose and ribulose determined by the cysteine–carbazole method. (2) One-tenth milliliter each of the column eluate and a 10 μmole/ml solution of NaRU5P were incubated 10 minutes at 37° C and a modified cysteine–carbazole reaction (12) carried out directly, the proportions of RU5P and XU5P being determined by the change in optical density due to XU5P formation. (3) One-tenth milliliter each of eluate and a NaRU5P solution (2.5 μmoles) were incubated 1 hour at 37° C. Acid phosphatase (0.1 ml), 0.1 *M* $MgCl_2$ (0.01 ml), and 0.1 *M* acetic acid to adjust the pH to about 5.0 with methyl red indicator were added and

the solutions incubated 1 hour at 37° C. They were concentrated to about 0.1 ml *in vacuo* over P_2O_5, a little Dowex 50 H^+ resin was added to remove cations, and the solution chromatographed using 90% phenol. After the chromatograms were dried and residual phenol extracted with diethyl ether RU (R_f 0.65) and XU (R_f 0.55) were detected by the trichloracetic acid – orcinol spray (21), followed by aniline phosphate (24) or aniline hydrogen phthalate (18) sprays.

The results obtained in this experiment are shown in Fig. 1. With PRI the results obtained with a second identical column are also shown to indicate the

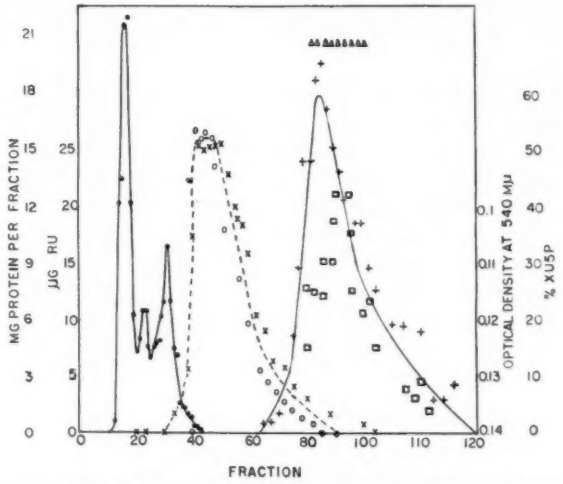

FIG. 1. Separation of PRI and EPIM using a DEAE column. Protein ●——●. PRI as determined by RU concentration (two different columns) ×- - -× and ○- - -○. EPIM as determined by % XU5P formed +——+; by decrease in O.D. at 540 mμ after 30 minutes at 37° C in the modified cysteine–carbazole reaction and recalculated on the basis of 0.1 μmole of RU5P giving O.D. of 0.15 and 0.1 μmole of XU5P, O.D. of 0.05 under these conditions (12) □——□; and by paper chromatography method as indicator of XU △ △ △.

close reproducibility obtained. No significant PRI or EPIM activity was found in the three major protein peaks emerging up to fraction 35, and these fractions accounted for 204 mg, or 86%, of the protein applied to the column.

PRI was obtained by pooling fractions 40–70 inclusive (146 ml). The preparation had 15 μg of protein per ml and a specific activity of 20.4 (Table I). If it is assumed that the original muscle (1.5 kg) had 2560 units of PRI, and the muscle protein content was 18%, then the specific activity would be roughly 0.01. On the basis of the original muscle the maximum purification attained in the procedures as outlined in Table I would then be about 2000 times.

Fractions 78–110 inclusive (Fig. 1) were pooled to yield 156 ml of EPIM preparation containing 5 μg of protein per ml. Since the purified enzyme is rather labile, all tests were carried out within 1 week at 0° C. A number of tubes containing 0.05 ml of EPIM plus 2 μmoles of NaRU5P in 0.02 ml were

incubated at 37° C and four tubes removed at intervals of 5, 10, 15, and 20 minutes for determination of XU5P formation by the cysteine–carbazole reaction after hydrolysis by acid phosphatase. The results showed that under these conditions 0.25 μg of protein (per sample) formed 0.0054 μmole of XU5P in 1 minute. Thus this preparation had a specific activity of 21.6. The recovery from 1.5 kg of muscle was 233 units as compared with 1330 units of PRI. While PRI proved very stable when frozen, EPIM preparations lost all measurable activity within 3 months at about −20° C.

Alternative Method for Preparation of PRI

The above DEAE column method provided a fairly satisfactory separation of PRI and EPIM. An alternative rapid procedure for preparing PRI free from EPIM is as follows:

In a typical experiment 43 ml of crude ammonium sulphate fraction containing 18.8 mg of protein per ml was heated to 55° and chilled as described above and centrifuged. The supernatant liquid was fractionated with $(NH_4)_2SO_4$ at pH 7.0, and the fraction precipitating between 0.6 and 0.75 saturation collected and dialyzed against frequent changes of demineralized water for 24 hours at 0° C to yield 8 ml of a preparation containing 42.5 mg/ml of protein. This preparation possessed a specific activity of 1.27, but the actual recovery was poor since much of the PRI remained in the fraction precipitating below 0.6 saturation with $(NH_4)_2SO_4$. This preparation was free from EPIM as determined by incubating samples with RU5P, dephosphorylating with phosphatase, and carrying out chromatography for RU and XU as described above. Only RU was found.

Preparation of RU5P

The barium was removed from BaR5P (2.6 mmoles) as described under Methods, and a solution of the potassium salt was obtained and incubated at pH 7.2 with 6.0 ml (7.6 units) of the PRI described in the preceding paragraph. The amount of RU5P formed was determined at intervals using the cysteine–carbazole reaction and the 1.85 factor. After 2.5 hours about 33% of the R5P was isomerized and, after a further 2 hours the reaction was complete with 43% RU5P formation. The solution was chilled to 0° C, deproteinized with 3 ml of 60% perchloric acid, and centrifuged 5 minutes at 10,000 *g*. The clear supernatant liquid was adjusted to pH 6.0 with 1 *N* KOH and the solution evaporated to dryness at 40° using a rotary evaporator. The dry residue was extracted at 0° C with water, the potassium perchlorate being removed by filtration. The clear filtrate and washings (35 ml) were treated with bromine water to remove aldopentose phosphate (24).

The clear colorless filtrate was run slowly onto a 12×2.8 (diam.) cm Dowex 1×10 formate column, washed with 100 ml of H_2O, and eluted with 0.1 *M* sodium formate in 0.1 *M* formic acid at 15 ml per minute, 14-ml fractions being collected. Fractions 41–56 inclusive, which contained practically all the cysteine–carbazole-positive material were pooled. The solution was adjusted to pH 5.0 with 1 *N* KOH, and then to pH 6.2 with warm, freshly filtered barium hydroxide solution. The BaRU5P was precipitated by adding four volumes of

absolute ethanol and the flocculent precipitate collected by centrifuging. The precipitate was dissolved in 50 ml of H_2O, a small amount of insoluble material removed by centrifuging, and the BaRU5P reprecipitated and dried in high vacuum over P_2O_5 at 0°. The yield was 250 mg of approximately 78% pure BaRU5P as judged by the cysteine–carbazole and orcinol reactions.

The product was dissolved in 3 ml of H_2O and run slowly through a 3×2 cm Dowex 50×8 H^+ column at about 3° C. The eluate and washings, after removal of insoluble material by centrifuging, were promptly adjusted to pH 6.2 with warm barium hydroxide solution, the BaRU5P precipitated and collected, and washed with 80% and absolute ethanol before being dried in high vacuum in P_2O_5 at 0° (yield 170 mg). The product gave 55.5 to 59% of the absorption of arabinose at 670 mμ in the orcinol reaction, 51% of the absorption of free RU after 2 hours at 37° C in the cysteine–carbazole reaction, was free from orthophosphate, and had 97% of the theoretical organic P content (assuming mol. wt. 365.5). It contained 7.0% alkali-stable pentose phosphate, the ratio of optical density 540/670 mμ was 0.33 in the orcinol reaction, and the specific rotation was $[\alpha]_D^{22}$ −20.2° (c=1.85 in 0.2 N HCl) based on the free acid (mol. wt. 230). This rotation compares favorably with that given by a fairly pure sample of L-RU5P; $[\alpha]_D^{20}$ +20° (25). Two zones, one characteristic of RU5P (2.5–3.0 cm from origin) and the other of R5P (6.0 cm from origin) resulted when the free acid was chromatographed. On the basis of these data the product contained about 90% BaRU5P and 7.0% R5P. This, or products of similar purity, were used in the following studies.

pH-Activity of PRI

Portions of a solution containing 10 μmole/ml of NaRU5P were either used without addition or were made 0.05 M with respect to glycine or sodium acetate. These were then adjusted to different pH values by cautious addition of traces of 1 N HCl or 10 N NaOH (Fig. 2). Duplicate 0.1-ml portions of these solutions were incubated 10 minutes with 0.02 ml of purified PRI (1 μg of protein) and the amount of RU5P formed was determined by the 2-hour cysteine–carbazole reaction and expressed as micrograms of RU. The results (Fig. 2) show that the enzyme has an optimum activity between about pH 7 and 8, loses its activity markedly between about pH 7 and 4, but has considerable activity at pH values between 8 and 11. A rather similar pH-activity pattern was observed for rabbit muscle PRI (24).

pH-Activity of EPIM

Purified EPIM containing 5 μg/ml of protein, and buffered with approximately 0.2 M Tris-HCl pH 7.3 as eluted from a DEAE column, was adjusted to lower pH values with very small amounts of 0.1 M acetic acid and to higher pH values with 1 N NaOH. Tubes containing 0.05 ml of buffered enzyme and 0.02 ml (0.19 μmole) of NaRU5P solution were incubated at 37° for 30 minutes. They were promptly chilled to 0° C and subjected to hydrolysis with acid phosphatase for 30 minutes at 37° under similar conditions to those described in the section dealing with isolation of the enzyme. The concentration of XU5P formed was determined by the cysteine–carbazole reaction (Method 1). The

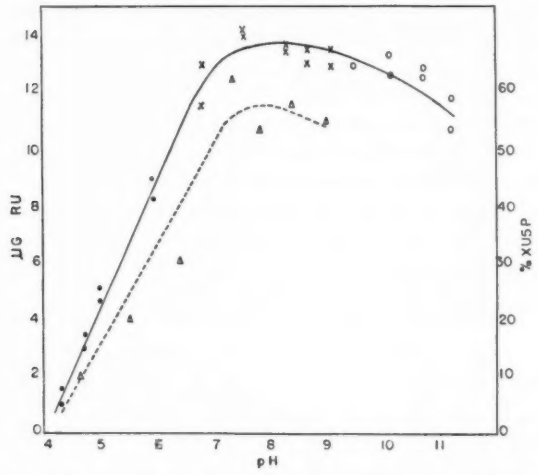

FIG. 2. pH–activity curves for PRI and EPIM. PRI with acetate ●——●, Tris ×——×, and glycine–NaOH ○——○ buffers. EPIM △- - -△ (see text for method used in adjusting pH).

results, shown in Fig. 2, indicate that the pH–activity curve of this enzyme is quite similar to that of PRI.

Equilibrium with Crude Preparations Containing both PRI and EPIM

Two preparations were studied, namely, the supernatant liquid from a crude enzyme which had been heated to 55° C and centrifuged (10.4 mg per ml of protein) and a similar preparation which had been lyophilized (35 mg per ml of protein). Duplicate portions of these preparations (0.01, 0.02, and 0.04 ml) were incubated at pH 7.2 for 10 minutes at 37° with 0.02 ml of a solution containing 0.18 µmole of NaR5P. The reaction was promptly stopped by immersing the tubes in a solid CO_2–acetone mixture, and the samples were subjected to analysis for total pentulose phosphate and XU5P content by the cysteine–carbazole method after prior hydrolysis with acid phosphatase. Similar results were obtained with the two enzyme preparations used and with the three concentrations. The total pentulose phosphate concentration varied between 50% and 58.5% (average 53.5%) of the initial amount of R5P used. The XU5P concentration varied between 52% and 59% (average 54%) of the total pentulose phosphate, and that of the RU5P between 41% and 48% (average 46%). The average R5P content (by difference) was 46.5%. The proportion of phosphate esters in this experiment, therefore, was about R5P, 46%; XU5P, 29%; RU5P, 25%.

A similar experiment was carried out using only 0.01 and 0.02 ml of the same two enzyme preparations and half the amount of R5P (0.095 µmole). In this instance the concentration of alkali-labile pentulose phosphate was determined. When the reaction was complete the samples contained from 54.5% to 56.6% (average 55.7%) of alkali-labile pentulose phosphate. In this

experiment the ratio was therefore: total pentulose phosphates, 55.7%:R5P, 44.3%, which is similar to that found in the preceding test.

Equilibrium with a Mixture of Purified PRI and EPIM

A mixture containing 19 μmoles of NaR5P, 2 ml of purified PRI (0.03 units), and 6 ml of EPIM (1.4 units) in a 10-ml volume was incubated at pH 7.2 and 37° C, duplicate 0.5-ml samples being removed at 10-, 30-, 45-, 60-, and 90-minute intervals for cysteine–carbazole determinations after dephosphorylation with acid phosphatase in the usual manner. The results showed that the total pentulose phosphate concentration increased up to 45 minutes, and that thereafter it remained approximately constant. The XU5P:RU5P ratio did not alter appreciably throughout the experiment, and the results obtained after 60 and 90 minutes showed that this ratio was 57% XU5P:43% RU5P, and that the total pentulose recovered was 1440 μg.

Alkali-stable pentose phosphate was also determined, using duplicate 0.15- and 0.20-ml aliquots of the reaction mixture after the 90-minute period. It was found that between 1080 and 1150 μg (av. 1110 μg) of alkali-stable pentose remained. Thus, the total recovery was 2550 μg of pentose plus pentulose, or 90% of the pentose of the R5P used. The ratio calculated from the above data was 43.0% R5P:32.5% XU5P:24.5% RU5P, which agrees well with that obtained with the crude mixed enzyme preparation in the preceding experiment.

Equilibrium with Purified PRI

Tubes containing 0.095 μmole of NaRU5P and 0.05 ml of a purified PRI preparation (1.2 μg of protein) were incubated at 37° C and the decrease in RU5P content was measured at intervals in one series by the cysteine–carbazole reaction and in another series by decrease in alkali-labile pentulose phosphate. A similar experiment was made except that NaR5P was used instead of RU5P. The results (Fig. 3) show that equilibrium was attained with 43–45% RU5P: 55–57% R5P.

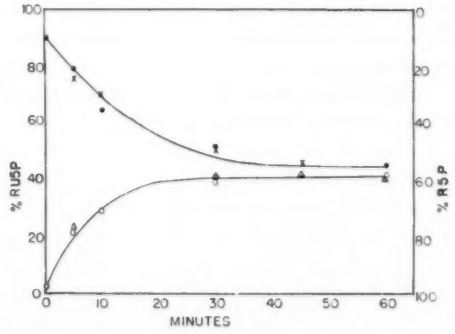

FIG. 3. Equilibrium with purified PRI. Enzyme plus RU5P, R5P formation determined by the cysteine–carbazole reaction ●——● and by increase in alkali-stable pentose ×——×. Enzyme plus R5P, RU5P formation determined by the cysteine–carbazole reaction ○——○ and by decrease in alkali-stable pentose △——△.

In a second experiment 0.5 mmole of NaR5P was incubated at 37° and pH 7.4 with 85 ml of PRI (2 mg protein = 41 units) in a total volume of 100 ml. To ensure equilibrium the solution was incubated 40 minutes. Analyses then showed 43.6% RU5P by measurement of alkali-labile pentulose, and 42.1% by the cysteine–carbazole reaction. The total pentose content by the orcinol reaction indicated a decrease of 22.5% at 670 mμ. This would account for the formation of 40% RU5P which gives about 57% of the optical density of R5P in this reaction. The average of these determinations indicated that the solution contained 42% RU5P and 58% R5P.

The solution was deproteinized at 5° C with 2 ml of 60% perchloric acid, centrifuged 5 minutes at 10,000 *g*, and the clear supernatant liquid adjusted to pH 5.5 with 1 *N* KOH. The solution was evaporated to dryness using a rotary evaporator at 37° C. The potassium perchlorate was largely removed from the crystalline residue by several extractions with water (1° C) followed by filtration. After adjusting the solution to pH 7.2 it was run onto a jacketed Dowex 1×10 formate column 35×1.8 (diam.) cm, and was eluted at 1° C at the rate of 3 ml/minute, first with 0.15 *M* ammonium formate containing 0.0005 *M* sodium borate, and then with 0.5 *M* sodium formate (24). The RU5P was removed with the first eluent (fractions 72–107) and the R5P (fractions 113–128) with the second eluent. The RU5P fraction (460 ml with washings) accounted for 34.4% of the R5P used, as determined by the cysteine–carbazole reaction using the 1.85 factor, and 35.4% as determined by optical density at 670 mμ in the orcinol reaction using the 1.76 factor. The second fraction (205 ml with washings) contained 49% of the R5P used as determined by the orcinol reaction. The recovery was therefore 84% of the R5P used and the ratio of RU5P:R5P was 1:1.5. Since further elution of the column yielded no material which gave positive cysteine–carbazole or orcinol reactions, it would appear that no significant amount of 3-ketopentulose 5-phosphate (12, 24) or xylose had formed.

A portion of each of the fractions was evaporated to a small volume at low temperature using a rotary evaporator, and the concentrates, after treatment with a little Dowex 50 H^+ resin to remove cations were chromatographed, using the *n*-propanol:ammonium hydroxide:water system referred to previously. Only single zones, characteristic of R5P and RU5P were found. The ratio of optical density at 540/670 mμ of the RU5P fraction in the orcinol reaction averaged 0.334, and that of the R5P fraction 0.19.

Equilibrium in a Crude Enzyme Preparation and Separation of Free XU and RU

Three millimoles of NaR5P were incubated with 15 ml of a lyophilized crude enzyme preparation (59 mg protein) in 250 ml total volume at pH 7.2. Analyses of duplicate 0.05-ml aliquots by the cysteine–carbazole method showed that the reaction neared completion in 20 minutes and was complete in 30 minutes with formation of 1.56 mmoles of pentulose phosphates (52% of the ribose in R5P used). The solution was deproteinized with perchloric acid and potassium perchlorate removed as in the preceding experiment, the R5P being removed by bromine treatment (24).

The clear solution (8.7 ml) was adjusted to pH 5.2 with 1 *N* KOH, and 0.8 ml of 1 *M* $MgCl_2$ and 5 ml of acid phosphatase were added. Testing of 0.02-ml aliquots at intervals showed that formation of orthophosphate (15) became constant after 2.5 hours, but that only 84% of the pentulose phosphates had apparently been hydrolyzed by this time. The total pentulose content of the solution was only 160 mg, or 81% of the orthophosphate liberated. At present no explanation can be offered for this discrepancy. Chromatography by ascending development in 90% phenol showed that the solution contained only ribulose (R_f 0.66) and xylulose (R_f 0.55), no other sugar being detected by the spray reagents used (18, 21, 22).

The solution was demineralized by passage through a mixed column of Dowex 50 H^+ and IR 45 OH^- resins, the eluate made 0.01 *M* with $Na_4B_2O_7$, and chromatographed on a 20×2.8 (diam.) cm Dowex 1×10 borate column under the conditions described by Dickens and Williamson (24). The fractions containing XU were pooled (1500 ml). Analyses showed that this fraction contained 58 mg of XU, the ratio of optical density 540/670 mμ in the orcinol reaction being 0.46, which agrees well with literature values (9, 18, 26). The xylulose solution was freed from cations, the boric acid removed, the solution decolorized with charcoal and evaporated to a syrup (24, 25). The xylulose was extracted with *n*-propanol to free it from insoluble material which could not be removed by mixed-bed ion exchange resin treatment. The specific rotation of the syrup, based on the xylulose content as determined by the cysteine–carbazole reaction was $[\alpha]_D^{23}$ −21.2° in water (*c*, 1.82), as compared with $[\alpha]_D^{18}$ −33.2° (5) and $[\alpha]_D^{25}$ −36.8° reported for pure D-xylulose (10). Ashwell and Hickman (12) reported a similar discrepancy with xylulose purified from Dowex 1 borate columns. Chromatography with 90% phenol revealed a single zone (R_f 0.67) characteristic of XU. The ratio of color development in 15′/120′ in the cysteine–carbazole reaction was 0.56, which is characteristic of xylulose. This evidence would indicate that the product was XU.

The fractions containing the RU were pooled to yield 1100 ml of solution which, as determined by the cysteine–carbazole reaction contained 38 mg of ribulose. In the cysteine–carbazole reaction color development was complete within 15 minutes, and the absorbancy ratio 540/670 mμ in the orcinol reaction was 0.86. Chromatography of the concentrated solution showed one component (R_f 0.56) which gave reactions typical of RU. These values are similar to those reported for RU by other investigators (9, 18, 26, 27).

The results of this experiment show a ratio of RU:XU of 1:1.5.

Inhibition of PRI by Phosphoribonic Acid

The sensitivity of spinach PRI to phosphoribonic acid was first observed by Axelrod and Jang (28), who noted 50% inhibition by a 1×10^{-4} concentration. In the present work purified PRI was also inhibited by phosphoribonic acid, the inhibition being about 45% by a 5×10^{-3} *M*, and 28% by 5×10^{-4} *M* concentration.

Equilibrium with Purified EPIM

A number of tubes containing 0.05 ml of purified EPIM (0.25 μg protein =

0.0055 units) were incubated with 0.02 ml (0.2 μmole) of NaRU5P at 37° C, four tubes being withdrawn at intervals to determine the XU5P concentration

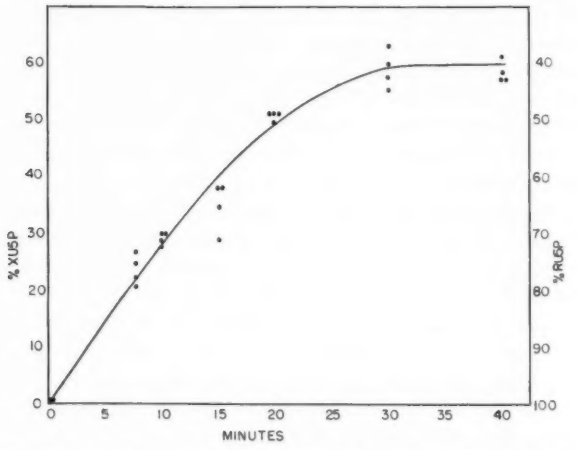

FIG. 4. Equilibrium with purified EPIM; XU5P formation determined by ratio of rate of color development after 15 and 120 minutes in the cysteine–carbazole reaction (see text).

by the cysteine–carbazole method. The results (Fig. 4) show that equilibrium is attained when the ratio of XU5P:RU5P is approximately 1.5:1.0.

Discussion

The equilibrium in the PRI system has been found by most investigators to be from 70–80% in favor of R5P formation and 20–30% in favor of RU5P formation (2, 25, 28, 29, 30, 31). On the other hand, the equilibrium with PRI prepared from erythrocytes was 41.5% RU5P:58.5% R5P, and from rabbit muscle 36–47% RU5P:53–64% R5P (24). In the present work the equilibrium with a fairly highly purified PRI from fish muscle has been found in different experiments to be 40–45% RU5P:55–60% R5P.

There has usually been quite close agreement in the results different investigators have obtained in studying equilibria with EPIM preparations from different sources, namely 40–45% RU5P: 55–60% XU5P (10, 24, 27, 32). On the other hand other investigators have reported ratios of 24–26% RU5P: 74–76% XU5P for EPIM preparations from spinach and muscle (31). In the present work with a fairly highly purified fish muscle EPIM the ratio was about 40% RU5P:60% XU5P. The same ratio has been reported for L-ribulose 5′-phosphate 4′-epimerase (33).

The equilibria in systems containing both PRI and EPIM have been reported as 35% R5P: 28% RU5P: 37% XU5P (12) and 41% R5P:14% RU5P:45% XU5P (31). The present work has shown that the equilibria obtained with a crude fish muscle preparation containing both PRI and EPIM, and with a

mixture of the fairly highly purified enzymes are similar; namely, 43.5–46% R5P:25.0–26.5 RU5P and 29–30.5% XU5P respectively. These results indicate somewhat higher proportions of both R5P and RU5P and smaller proportions of XU5P than those previously recorded for this system. However, the ratio of XU5P to R5P found in the present work with both mixed purified enzymes and the crude enzyme mixture (pentulose phosphates 53.5–55.7%: R5P 40.5%–44.3%) is very similar to the average values reported for mixed rabbit muscle PRI and EPIM, namely 58.5% mixed pentulose phosphates: 41.5% R5P (24).

The possible reasons for the apparent discrepancies in the ratios of reactants in the above systems have been discussed (31, 32) but so far there has been no really satisfactory explanation for them.

Acknowledgment

I wish to thank Mr. A. P. Ronald for making the specific rotation determinations.

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ROLE OF PHOSPHOLIPIDS IN MITOCHONDRIAL RESPIRATION[1]

E. PETRUSHKA, J. H. QUASTEL, AND P. G. SCHOLEFIELD[2]

Abstract

The addition of heated snake venom solutions to suspensions of rat liver, kidney, or brain mitochondria results in an initial stimulation of the rate of respiration, which is approximately the same for a variety of substrates, followed by a rapid decline.

The presence of phospholipase A in heated snake venom is demonstrated by the formation of lysolecithin from mitochondrial lecithin. Various phospholipids, when added to mitochondria, have a protective influence against the inhibitory effects of phospholipase of heated venoms on respiration. *Clostridium welchii* toxin, which contains phospholipase C, has an effect on mitochondrial respiration which is similar to that of venom phospholipase A but the addition of phospholipid has no protective effect.

Glutathione exercises a protective action against the inhibitory effects of phospholipase A on rat liver or kidney mitochondrial respiration, the action consisting of a prolongation of the initial stimulated phase of respiration. The effect does not seem to obtain with rat brain mitochondria.

Exposure of rat brain cortex slices, but not those of rat kidney or liver, to heated venom results in changes of respiratory rates similar to those obtained with brain mitochondria.

Studies of the effects of phospholipases on the respiratory metabolism of animal tissues, examined in the form of homogenates, minces or subcellular fractions, have been the subjects of a variety of investigations (1–10). A convenient source of phospholipase A is snake venom (11–13) and indeed Slotta and Fraenkel-Conrat (14) have reported the isolation of a neurotoxin from *Crotalus terrificus terrificus* in crystalline form, termed crotoxin, which has phospholipase activity. A characteristic feature of phospholipase A (lecithinase) is its relative thermostability. Its ability to withstand heating to 100° C for 15 minutes was utilized by Braganca and Quastel (1, 2) in their studies of the effects of heated snake venoms on a number of enzyme systems. These authors concluded that the highly inhibitory effects of venom phospholipase A on a variety of enzyme systems (e.g. succinic oxidase, cytochrome oxidase, choline oxidase, pyruvic oxidase) is due to degradation of mitochondrial structures on whose integrity the enzymes concerned depend for their optimal activities. The inactivation of the succinic oxidase system by phospholipase has been confirmed by Neumann (15) and by Radomski and Deichmann (16), who have used protein fractions separated by electrophoresis.

A product of phospholipase A activity, lysolecithin, may be implicated in the suppression of enzyme activities brought about by phospholipase. It has been pointed out (17, 18) that lysolecithin has the property of clearing opaque suspensions of brain homogenates and affects a number of enzymes, e.g. acid phosphatase and monoamine oxidase. Morrison and Zamecnik (19) have, in fact, suggested that lysolecithin, if formed in the central nervous system, may

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produce a local demyelination. Mitochondria have a high content of phospholipid (20) and are unstable in the presence of phospholipase and lysolecithin (7). Lysolecithin and other lipids are destructive to liver mitochondria (21) and Witter, Morrison, and Shepardson (22) have concluded that lysolecithin can uncouple oxidation from phosphorylation in rat liver mitochondria oxidizing succinate or β-hydroxybutyrate and can depress the rates of oxidation of these substances. Lysolecithin apparently increases the activity of cytochrome oxidase (22) but phospholipase A inactivates the enzyme (1, 2) a result which is in agreement with the observation (23) that cobra venom inhibits cytochrome oxidase.

Phospholipases can affect tissue respiratory activities independently of the formation of lysolecithin. It has been shown (24) that phospholipase C (lecithinase C or the alpha-toxin of *Clostridium welchii*), which does not form lysolecithin, inhibits the aerobic oxidation of succinate by suspensions of guinea pig intestine and other tissues, though neither succinic dehydrogenase nor cytochrome oxidase seems to be affected. MacFarlane (25) found, however, that cytochrome oxidase is completely inhibited by phospholipase C. In this connection it should be noted that Ball and Cooper (26) have suggested that mitochondrial phospholipids may serve as cement substance for interacting enzyme groups such as those present in the succinoxidase system. Moreover, it has been shown (3) that when approximately 10% of the hydrolyzable lecithin of mitochondria is removed by lecithinase A, and before succinic dehydrogenase and cytochrome oxidase are impaired, the succinoxidase complex is rendered inactive. This finding is compatible with the observations (3, 6, 16) that succinic dehydrogenase and cytochrome oxidase are only partially affected even at relatively high phospholipase concentrations. Thus a component of the succinoxidase system, other than the dehydrogenase itself, is unstable in the presence of phospholipase A or C although it is known (2) that the presence of succinate protects the enzyme to some extent against the action of phospholipase A.

The present investigations have been carried out to throw further light on the mode of action of phospholipases on mitochondrial metabolism.

Materials and Methods

Animals.—Male Wistar rats weighing 200–250 g were used throughout the work described in this paper.

Mitochondria.—These were isolated by the method of Schneider (27) for liver and kidney, and by the method of Brody and Bain (28) for brain. In all cases the homogenizing medium consisted of a mixture of 8 ml 0.25 *M* sucrose and 2 ml 0.15 *M* sodium bicarbonate. Mitochondria obtained from 1 g liver, 420 mg kidney, or 450 mg brain were used per manometric vessel throughout for respiratory studies and approximately 20% less than these amounts for studies of oxidative phosphorylation.

Respiratory activity.—This was measured with the conventional Warburg apparatus at 37° in an atmosphere of air for mitochondria and oxygen for

tissue slices. The latter were cut by means of a Stadie–Riggs microtome and were carefully maintained at a temperature of 0–5° prior to incubation.

Incubation media.—Tissue slices were incubated in a Krebs–Ringer–phosphate medium, and mitochondria in the medium described by Judah and Rees (29) as previously modified (Scholefield (30)). The final volume, in all cases, was 3 ml.

Heated venoms.—Three snake venoms were used. *Agkistridon piscivorus* (A.P.) venom was obtained from Ross Allen's Reptile Institute, Silver Springs, Florida; Russel's Viper (R.V.) venom from the Wellcome Research Laboratories, and the *Naja naja* (N.N.) cobra venom, a gift from the Haffkine Institute, Bombay. Preparations of heated venoms were obtained by placing a solution of the venom in a boiling water bath for 10 minutes, centrifuging, and combining the supernatant with one washing solution. The volume was then made up with water so that the concentration was equivalent to 7.5 μg/ml of the original lyophilized venom. The solution generally contained approximately 2.5 μg/ml of protein, determined as nitrogen. Such solutions displayed no loss of activity if stored at $-20°$ C. Aliquots were stored separately and, once thawed, were maintained at a temperature of 0–3° C. All values quoted refer to the original venom content of such solutions before heating.

Clostridium welchii lecithinase.—This was obtained in a crude form from Dr. V. Fredette, Institut de Microbiologie, University of Montreal. This material was dialyzed against running tap water for 6 hours and then lyophilized. It was stored in a vacuum desiccator and made up to a final concentration of 50 mouse units/ml as desired.

Egg lecithin.—This was prepared as described by Pangborn (31).

Lysolecithin.—This was prepared from lecithin by the method of Kates (32). Chromatography of the product on silica-impregnated paper (33, 34) indicated that it contained both lecithin and lysolecithin.

Results

The Effects of Heated Venom on the Rate of Mitochondrial Respiration

When heated *Agkistridon piscivorus* (A.P.) venom is added to preparations of rat liver mitochondria oxidizing pyruvate and fumarate, the results shown in Fig. 1 are obtained. It is apparent that addition of the heated venom results in an initial stimulation of respiration which is approximately 50%. Within the range of venom concentrations added (75–750 μg/3 ml) this increased rate of respiration remains constant. Later, there is a sudden decline in respiratory activity. The interval between the time of the addition of the heated venom and the time at which the sudden decrease in respiratory activity occurs is termed the "fall-off" time. The magnitude of the "fall-off" time depends on the amount of heated venom added. In the example illustrated in Fig. 1 the rapid decline in respiratory activity occurs 50 minutes after the addition of 225 μg heated venom and 30 minutes after the addition of 450 μg heated venom. When 100 μg heated venom is added, the stimulated phase of respiration may last for more than 2 hours. Addition of quantities of heated venom larger than

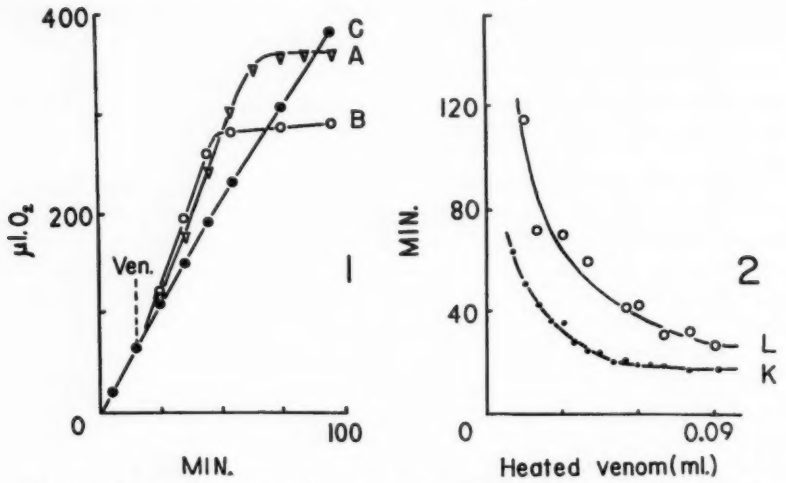

FIG. 1. The effect of heated A.P. venom on the oxidation of sodium pyruvate (6.7 m*M*) and potassium fumarate (0.67 m*M*) by rat liver mitochondria. The following amounts of venom were added after 15 minutes: A, 225 µg; B, 450 µg; C, nil.
Ordinate = µl oxygen consumed. Abscissa = Time in minutes.

FIG. 2. The effect of variation in the amount of heated A.P. venom solution on the "fall-off" time. The original venom solution contained 7.5 mg/ml. L — liver mitochondria. K — kidney mitochondria.
Ordinate = "Fall-off" time in minutes. Abscissa = ml heated AP venom present in manometer vessel.

650 µg/vessel to liver mitochondria does not result in a decrease of the value of the "fall-off" time below about 20 minutes (see Fig. 2). Qualitatively similar results to these are obtained using heated Russel's Viper venom or heated cobra venom. These phenomena are also seen with kidney mitochondria in the presence of pyruvate and malate but when rat brain mitochondria are employed there is not a complete loss of respiratory activity. There is still an initial marked stimulation of respiratory activity followed by a more gradual decrease, but the final rate of respiration attained is approximately 50% of the maximum, in contrast to the complete loss of respiratory activity observed with liver or kidney mitochondria.

Effects of Heated Venom on Mitochondrial Respiration in Presence of Various Substrates

The effects of heated venom on the mitochondrial oxidation of various substrates are shown in the results recorded in Tables I and II. The percentage stimulation of the oxidation of the longer chain fatty acids and the "fall-off" time were approximately the same in magnitude as the corresponding values observed for the endogenous activity but with all other substrates tested, except choline, the initial stimulation was of the order of 50%. With choline the initial stimulation was 76%. The "fall-off" time in the presence of the components of the citric acid cycle was approximately 70 minutes after the addition of heated venom but in the presence of the shorter chain fatty acids

the "fall-off" time was only 25–30 minutes, i.e., it was only slightly greater than that observed for the longer chain fatty acids and for the slight endogenous activity.

TABLE I

The effect of heated venom on the oxygen consumption of rat liver mitochondria in the presence of various substrates

Substrate (6.7 m*M*)	μl oxygen uptake (10–30 min)		Initial percentage stimulation	"Fall-off" time (min)
	In the absence of venom	In the presence of venom		
Nil	35	41	19	20
Citrate	136	199	44	74
α-Ketoglutarate	124	164	43	81
Succinate	124	185	48	69
Fumarate	115	168	49	69
Malate	126	185	46	72
Pyruvate + malate	118	188	58	67
Choline chloride	65	112	76	43

The acids employed as substrates were added as their sodium salts; the concentration of malate was 0.67 m*M* when pyruvate was also used as substrate. A.P. venom (300 μg) was added as indicated in the table. The oxygen uptakes were measured in the interval between 10 and 30 minutes after the addition of the venom. Figures quoted are the mean values obtained from three separate experiments. The "fall-off" time was the interval between the addition of the venom and the time corresponding to the sudden decrease in respiratory activity.

TABLE II

The effect of heated venom on the oxygen consumption of rat liver mitochondria in the presence of various fatty acids

Substrate (2.67 m*M*)	μl oxygen uptake (10–20 min)		Initial percentage stimulation	"Fall-off" time (min)
	In the absence of venom	In the presence of venom		
Nil	17	21	24	29
β-Hydroxybutyrate	28	41	35	35
Octanoate	65	96	48	28
Myristate	39	59	51	25
Laurate	36	51	42	29
Palmitate	57	67	18	25
Stearate	44	50	14	22
Oleate	55	64	18	24

The conditions were the same as those described under Table I except that the substrate concentrations were reduced to 2.67 m*M* and the oxygen uptake, because of the early cessation of respiratory activity, could only be measured over the interval 10–20 minutes after tipping of 300 μg A.P. venom.

Effects of Heated Venom on the Mitochondrial Oxidation of Pyruvate-2-C^{14}

The possibility that the stimulation of respiration is due to the oxidation of fatty acid liberated by the action of phospholipase A was considered. However, this seemed unlikely in view of the significant stimulation of the respiratory activity of brain mitochondria by heated venom in spite of the low rate of fatty acid oxidation by these mitochondria. In order to investigate the nature of the stimulation of respiration of rat liver mitochondria, pyruvate-2-C^{14} was employed as substrate and its rate of oxidation to $C^{14}O_2$ was measured. Liver mitochondria were incubated with and without 150 μg heated A.P. venom in the presence of 6.7 m*M* pyruvate-2-C^{14} and 0.67 m*M* malate. The oxygen

uptake and the $C^{14}O_2$ produced in 40 minutes were estimated both in the presence and absence of the heated venom. An increased $C^{14}O_2$ production of 49% was observed (2276 counts/minute increased to 3391 counts/minute), which was comparable with the increase in respiratory activity of 42% (198 μl oxygen taken up in the absence of heated venom and 283 μl oxygen taken up in its presence). These results make it likely that the increased rate of respiration of mitrochondria oxidizing pyruvate, and the intermediates of the citric acid cycle, is connected with an increased rate of oxidation of the acetyl groups derived from pyruvate.

Phospholipase A in Heated Venom

The presence of phospholipase A in heated venom was demonstrated by the procedure described by Braganca and Quastel (2) in which the enzyme is incubated with its substrate in a bicarbonate medium and the rate of carbon dioxide evolution measured. Heated mitochondria, when used as substrate, also give rise to an evolution of carbon dioxide. When unheated (i.e. respiring) mitochondria are incubated with heated venom and the phospholipids separated (34) and chromatographed (33, 35), it is evident that, as a result of incubation, the spot corresponding to lecithin grows faint and a new spot corresponding to lysolecithin appears.

Effects of Lecithin and Mitochondrial Components

It has previously been shown by us (9) that purified egg lecithin protects liver mitochondria from the inhibitory action of heated venom. Similarly, Tookey and Balls (8) found that soybean lecithin protects mitochondrial succinoxidase from the action of phospholipase D.

It may be seen from the results given in Table III that lecithin has little effect on mitochondrial respiration in the absence of heated venom or on the initial stimulation of respiration by the heated venom but it delays the eventual decrease in respiratory activity brought about by the heated venom.

TABLE III

The effects of heated venom and egg lecithin on the respiratory activity of rat liver mitochondria

mg lecithin added	μg heated A.P. venom added	μl oxygen taken up in 60 min	"Fall-off" time (minutes)
0	0	291	—
	225	243	30
2	0	—	—
	225	286	39
4	0	276	—
	225	327	44
6	0	—	—
	225	349	49
8	0	275	—
	225	369	52
10	0	—	—
	225	384	56

The substrates used were 6.7 m*M* pyruvate and 0.67 m*M* malate. Standard manometric conditions were employed.

As heated mitochondria contain a substrate for the phospholipase A of heated venom, their ability to protect unheated mitochondria from the action of heated venom was examined. It is evident from the results quoted in Table IV that this preparation has a depressing effect on respiratory activity, which

TABLE IV

The effects of heated venom and heated liver mitochondria on the respiratory activity of rat liver mitochondria

	ml heated mitochondria			
	0	0	0.25	0.25
	μg heated venom			
	0	225	0	225
0 – 10 min	65*	71	39	77
10 – 20 min	69	20	37	82
20 – 30 min	65	14	36	39
30 – 40 min	60	8	37	13

*All values refer to μl oxygen taken up in the interval indicated. The substrate was 6.7 m*M* pyruvate and 0.67 m*M* malate. Standard manometric conditions were employed. The concentration of heated mitochondria was the same as that of the unheated mitochondria.

may be due to the action of breakdown products of mitochondrial phospholipid formed on heating (36). It is noteworthy, however, that the time of onset of the inhibitory effect of heated venom on mitochondrial respiration is retarded by the presence of the heated mitochondria.

The Effects of Lysolecithin and C. welchii Toxin

The effects of phospholipase A on mitochondrial respiration may be due to the formation of lysolecithin or they may be due to removal of phospholipid essential for the mitochondrial enzyme activity. These two alternatives were investigated.

A sample of lysolecithin was prepared by incubating lecithin with heated venom in the presence of calcium ions and ether vapor (32). The effect of this material, containing lysolecithin, heated venom, and the remaining unhydrolyzed lecithin, on the respiration of rat liver mitochondria was then compared with that of an equivalent amount of either heated venom, lecithin alone, or heated venom plus lecithin. It is apparent from the results obtained (Table V) that the material formed after preincubation was more effective than the heated venom alone or of a mixture of lecithin, ether, and calcium ions in influencing mitochondrial respiration. Although the lysolecithin production leads to a decrease in the "fall-off" time it does not, within experimental error, increase the initial stimulation. These results indicate that lysolecithin formation may account partly for the action of heated venom on mitochondrial respiration.

In view of the demonstration (25, 37) that the alpha toxin (lecithinase C) of *C. welchii* inhibits succinoxidase, simultaneously releasing phosphorylcholine but not lysolecithin from the phospholipids present, the action of this toxin on mitochondrial respiration was investigated. The results, which are sum-

TABLE V

The effects of heated venom and lysolecithin on the respiratory activity of rat liver mitochondria

Interval	No additions	Control	Venom only	Preincubation time, min 3	6	15	25	35	45
0 – 20 min	94	93	200	217	224	217	146	128	93
20 – 40 min	106	95	180	148	106	52	14	13	16
40 – 60 min	108	95	25	17	10	4	0	0	0
"Fall-off" time (min)	—	—	38	33	30	22	14	12	8

The additions are abbreviated as follows: Venom = 225 μg R.V. heated venom; control = preincubation mixture without venom; 3 minutes, 6 minutes, etc., preincubation mixture containing 225 μg venom, lecithin, calcium ions, and ether incubated for the designated number of minutes by the method of Kates (32). All values are μl of oxygen taken up in the indicated interval under the standard manometric conditions in the presence of 6.7 m*M* sodium pyruvate and 0.67 m*M* sodium malate.

marized in Table VI, indicate that up to a concentration of approximately 1.5 mouse units/ml the alpha toxin brings about an increased rate of respiration and the respiratory activity does not decline until after about two hours under the given experimental conditions. With increase in the concentration of alpha toxin there is a gradual decrease in the "fall-off" time, the final value of which is approximately 15 minutes. There is, clearly, a definite similarity between the results obtained with phospholipase C and venom phospholipase A so far as their effects on mitochondrial respiration are concerned.

TABLE VI

The effect of *C. welchii* phospholipase C on the respiratory activity of rat liver mitochondria

Phospholipase C added (mouse units)	Initial respiratory activity (μl oxygen taken up in 10 minutes)		"Fall-off" time (in minutes)
0	47		—
0.5	46	(0)	>120
1.5	53	(13)	>120
2.5	65	(38)	>120
4.5	73	(55)	68
5.5	67	(43)	30
7.5	50	(6)	18

The figures in parentheses refer to percentage stimulation. Standard manometric conditions were employed throughout. The substrate was 6.7 m*M* pyruvate and 0.67 m*M* malate.

The effect of the alpha toxin in the presence of purified egg lecithin was then investigated in order to ascertain whether egg lecithin gives a protective action similar to that obtained against heated venom. The results obtained are shown in Table VII. It is apparent that no significant protection is afforded by the egg lecithin. There was in fact a tendency for the respiratory activity of the mitochondria to decline a little earlier in the presence of lecithin. It is possible that the diglyceride, produced from the phospholipid by the action of the phospholipase C, may be responsible for this slight inhibitory effect (36).

The Effects of Glutathione

Numerous reports have indicated that venom lecithinase A is sensitive to sulphydryl compounds. Reduced glutathione considerably reduces the toxicity

TABLE VII

The effect of *C. welchii* phospholipase C and egg lecithin on the respiratory activity of rat liver mitochondria

	Egg lecithin (mg)								
	0	4	8	0	4	8	0	4	8
	Phospholipase C (mouse units)								
	0	0	0	2.5	2.5	2.5	5.0	5.0	5.0
0 – 30 minutes	158	147	142	200	197	240	157	144	132
30 – 60 minutes	161	161	149	199	188	197	67	36	12
60 – 90 minutes	157	152	141	179	155	173	16	15	17

All values refer to μl oxygen taken up in the interval indicated. Standard manometric conditions were employed, the substrate being 6.7 m*M* pyruvate and 0.67 m*M* malate.

of cobra venom to animals (38, 39), and it is to be noted that crystalline crotoxin contains 4% sulphur, 87% of which is present as S—S linkages (14, 40, 41). On the other hand, De (42) has shown that the haemolytic activity, removed from crude venom by treatment with oxidizing agents, may be restored by treatment with glutathione, cysteine, or hydrogen sulphide. The influence of glutathione on the action of heated venom on respiring mitochondria was therefore investigated.

Results given in Table VIII show that glutathione affords rat liver mitochondria marked protection against the inhibitory action of the heated venom.

TABLE VIII

The effects of heated venom and glutathione (GSH) on the rate of oxidation of sodium pyruvate by rat liver mitochondria

Time interval	μg heated A.P. venom added	μmoles GSH added				
		0	0.2	2	10	20
0 – 30 min	0	90	92	98	102	105
	300	159	158	178	179	188
30 – 60 min	0	88	86	84	89	93
	300	66	49	68	179	159
60 – 90 min	0	80	80	80	78	83
	300	3	2	28	135	147
90 – 120 min	0	75	72	70	76	82
	300	3	2	6	30	120

All values refer to μl oxygen taken up during the period indicated following the addition of heated venom to rat liver mitochondria respiring in the presence of 6.7 m*M* pyruvate and 0.67 m*M* malate under standard manometric conditions.

A similar protective action is observed when rat kidney mitochondria are used. In both cases the protective action consists of a prolongation of the initial stimulated phase of respiration, the prolongation being directly proportional to the amount of glutathione added. Liver mitochondria are more easily protected than kidney or brain mitochondria, 20 μmoles glutathione being sufficient to increase the "fall-off" time to more than two hours in the presence of 300 μg heated A.P. venom. With kidney mitochondria, when only 225 μg heated A.P. venom were added, 60 μmoles glutathione failed to increase the

"fall-off" time to more than 90 minutes. With brain mitochondria glutathione elicits a slight stimulation (15%) of respiration, which is apparently independent of that produced by the heated venom, and affords no protection against the inhibitory action of the venom. Braganca and Quastel (2) also observed that glutathione (10 m*M*) does not influence the inhibitory action of heated cobra venom on the succinoxidase system of rat brain homogenate.

The Effects of Heated Venom on Other Types of Cell Preparations

In view of the marked effects of heated venoms on mitochondrial preparations, experiments were carried out to determine the effect, if any, of heated venoms on tissue slices or intact cells. Some of the results obtained are quoted in Table IX. There is no inhibitory effect of relatively high concentrations of

TABLE IX

The effects of heated venom on the respiratory activity of tissue slices

	Liver		Kidney		Brain	
Time interval	No venom added	750 μg R.V. venom added	No venom added	750 μg R.V. venom added	No venom added	750 μg R.V. venom added
Before additions						
0 – 30 min	8.3*	10.0	22.4	22.5	5.5	5.5
After additions						
0 – 30 min	7.0	8.0	23.2	26.0	5.4	6.9
30 – 60 min	6.1	7.4			5.2	5.2
60 – 90 min	—	—			5.7	2.7

Medium as described under Methods and Materials, temperature 37° C and time as indicated. Substrate 0.01 *M* sodium pyruvate.

*All the values quoted are Q_{O_2} values (μl O_2 consumed per hour per mg dry weight tissue) measured over the intervals indicated.

heated venom on the respiratory activities of rat liver or kidney slices. With brain cortex slices, however, a significant initial stimulation of the respiration of brain slices with a subsequent marked decrease in respiratory activity takes place. A similar effect on the anaerobic glycolysis of the brain slices also occurs. No effect is observed when slices of mouse sarcoma S37 or the corresponding host liver are employed nor is there any apparent effect on the respiration of Ehrlich ascites carcinoma cells in the presence or absence of pyruvate. On the other hand, there is a very marked inhibitory effect on the succinoxidase and choline oxidase activities of liver cells isolated by the method of Anderson (43).

TABLE X

The effect of heated R.V. venom on the succinoxidase activity of isolated liver cells

μg heated R.V. venom added	μl oxygen taken up in 25 minutes	μg heated R.V. venom added	μl oxygen taken up in 25 minutes
0	114	15.0	75
3.75	106	22.5	62
7.5	98	37.5	41
11.3	89	75.0	23

The cells were isolated as described by Anderson (43) and incubated in Krebs–Ringer–phosphate medium in Warburg flasks in air at 37° C. The substrate was 25 m*M* succinate.

It should be noted that at no time is there any stimulatory effect of heated venom on the respiratory activities of these cells. Some typical effects of heated R.V. venom on the succinoxidase activity are quoted in Table X. It seems surprising that the isolated liver cells, which cannot perform the oxidative steps for which the phospholipids are so essential, are so sensitive to the venom phospholipase, particularly as the liver slice is resistant to the action of heated venoms. Microscopic examination confirms the visual indication that the cells had undergone rupture or lysis.

Discussion

When heated snake venom, which retains its phospholipase A activity, is added to mitochondria of rat liver, kidney, or brain there is an immediate increase in the rates of oxygen uptake. The increase usually amounts to about 50% but the increase may be a little higher, as with choline when it amounts to over 70%, or lower as with laurate, palmitate, and stearate when it amounts to approximately 15%. An increase in cytochrome oxidase activity, using ascorbic acid as the reducing agent, on addition of crotoxin (phospholipase A) was noted by Nygaard and Sumner (3). The extent of stimulation seems to be independent of the concentration of heated venom added within the limits of 25–250 μg heated venom/ml. Experiments with pyruvate-2-C^{14} have shown that the increased respiratory activity is associated with oxidation of the added substrate and not with the release and oxidation of endogenous substrate.

Subsequently there is a diminution in the respiratory rate and in the cases of rat liver and rat kidney mitochondria the rate of respiration becomes nil. The loss of respiratory activity when heated venom is added to brain mitochondria is only of the order of 50%.

The active component in heated venom has been shown to be the phospholipase A. Chromatographic evidence has shown that, under the incubation conditions employed, lecithin disappears from the mitochondria and lysolecithin appears in its place. When phospholipase A is replaced by phospholipase C (from *C. welchii*) qualitatively similar effects on respiratory activity are obtained. It is therefore concluded that the active component in heated venom is the phospholipase A which is present and that some, at least, of the observed effects must be due to removal of phospholipid through the action of this enzyme. The mechanism of the effects will be discussed more fully in the following paper.

Glutathione exercises a remarkable protective action against the inhibitory effects of phospholipase A on rat liver or rat kidney mitochondrial respiration, the action consisting of a prolongation of the initial stimulated phase of respiration. The protective action is not apparent with rat brain mitochondria. The phenomenon points to the possibility that certain mitochondrial components require for their integrity free —SH groups, which are affected by phospholipase A with resultant disintegration of these components. It is to be recalled that crotoxin is rich in —S—S— groups that may well oxidize free —SH groups in the mitochondria. The presence of glutathione may protect the relevant

thiol components in mitochondria in a manner similar to its well-known protective effect on the thiol groups of succinic dehydrogenase. No protective action of glutathione, however, is observed on the P/O ratio (see following paper), indicating that its effect is not associated with the lipids which are first attacked by phospholipase A and which appear to be essential for the process of oxidative phosphorylation.

Exposure to heated venom of rat brain cortex slices but not rat kidney or liver slices in presence of glucose results in changes of respiratory rates very similar to those obtained with brain mitochondria. There is an initial rise in the rate of oxygen consumption followed by a fall. The implication is that brain cell membranes contain phospholipid groups that may be attacked by venom phospholipase and that metabolic activity associated with, or controlled by, the membranes is thereby affected (9). Such groups appear not to be present in the same amount in the cell membranes of liver slices or the membranes of the tumor tissues investigated or, if they are present, they are not associated with respiratory activity. Yet the respiratory activities of isolated liver cells are affected by heated venom perhaps because the isolated liver cells offer more surface structures for attack by the phospholipase than those available in liver slices.

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ROLE OF PHOSPHOLIPIDS IN OXIDATIVE PHOSPHORYLATION AND MITOCHONDRIAL STRUCTURE[1]

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Abstract

The addition of small amounts (50 μg protein) of heated snake venom to respiring preparations of mitochondria from rat liver or kidney brings about an uncoupling of oxidation from phosphorylation as measured by a fall in the P/O ratio. When larger amounts (250 μg protein) of heated venom are added a rapid decline in the respiratory activity eventually takes place which coincides with a breakdown in the mitochondrial structure. On addition of smaller amounts (e.g. 7.5 μg protein) of heated venom to liver or kidney mitochondria there occurs a release of soluble proteins including enzymes, e.g. ATP-ase.

Various phospholipids are able to exert a specific protective effect against the uncoupling action of heated venom. Albumin exhibits a non-specific effect but glutathionine, which helps to maintain the respiratory activity of mitochondria in the presence of heated venom, does not prevent the accompanying fall in the P/O ratio.

The importance of the structural integrity of mitochondria for their enzymic activities is discussed, special reference being made to the role of phospholipids.

It was shown in the previous paper (1) that phospholipids can influence the course of the interaction between heated snake venom (phospholipase A) and respiring mitochondria derived from various tissues of the rat.

Habermann (2) concluded that oxidative phosphorylation of liver homogenates is affected by venoms and that lipids may be a significant factor in this phenomenon. Petrushka, Quastel, and Scholefield (3) showed that one of the first effects of the addition of venom phospholipase to rat liver mitochondria is a marked decrease of the P/O ratio, which may fall to zero even before the rate of oxygen consumption by the mitochondria is decreased. They concluded that the first substrate in the mitochondria to be attacked by the venom phospholipase is a phospholipid involved in the mechanism of coupling of oxidation with phosphorylation. Hydrolysis of this phospholipid results in the elimination of phosphorylation without necessarily disturbing respiratory activity. The phospholipase may then affect other phospholipids that are either constituent parts of the respiratory enzymes or which act as a medium that binds the enzymes together in the particules, thus allowing electron transport to take place.

The present experiments have been designed to yield further information concerning the role of phospholipids in oxidative phosphorylation and in the maintenance of mitochondrial structure.

Materials and Methods

Animals.—Male Wistar rats weighing 200–250 g were used throughout the work described in this paper.

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Mitochondria.—These were isolated by the method of Schneider (4) for liver and kidney, and by the method of Brody and Bain (5) for brain. In all cases the homogenizing medium consisted of a mixture of 8 ml 0.25 *M* sucrose and 2 ml 0.15 *M* sodium bicarbonate. Mitochondria obtained from 1 g liver, 420 mg kidney, or 450 mg brain were used per manometric vessel throughout for respiratory studies and approximately 20% less than these amounts for studies of oxidative phosphorylation.

Respiratory activity.—This was measured with the conventional Warburg apparatus at 37° in an atmosphere of air.

Incubation media.—Mitochondria were incubated in the medium described by Judah and Rees (6) as previously modified (Scholefield (7)). The final volume, in all cases, was 3 ml.

Oxidative phosphorylation.—This was measured as previously described (8). Mitochondria were incubated in the standard incubation medium in the presence of 0.01 *M* fluoride and the reaction was begun by tipping into the main compartment 0.2 ml 0.3 *M* glucose solution containing 1 mg hexokinase (Sigma). After 10 minutes' incubation the flasks were quickly removed, placed on cracked ice, and the reaction terminated by addition of 1 ml 15% trichloroacetic acid. After centrifugation, phosphate determinations were made on 0.2-ml aliquots of the supernatants using the method of Fiske and Subbarow (9).

Heated venoms.—Three snake venoms were used. *Agkistridon piscivorus* (A.P.) venom was obtained from Ross Allen's Reptile Institute, Silver Springs, Florida; Russel's Viper (R.V.) venom from the Wellcome Research Laboratories, and the *Naja naja* (N.N.) cobra venom, a gift from the Haffkine Institute, Bombay. Preparations of heated venoms were made as previously described (1).

Egg lecithin.—This was prepared as described by Pangborn (10).

Lysolecithin.—Lysolecithin was prepared from lecithin by the method of Kates (11). Chromatography of the product on silica-impregnated paper (12, 13) indicated that it contained both lecithin and lysolecithin.

Results

The Effects of Lecithin and Albumin on the Action of Heated Venom on Oxidative Phosphorylation by Mitochondria

Phospholipids are now known to be implicated in the phenomena connected with the uncoupling of oxidation from phosphorylation (2, 3, 14). It was, therefore, of interest to determine whether lecithin can protect mitochondria from the effects of heated venom on phosphorylation as well as on respiration. A non-specific protection against the effects of uncoupling agents by serum albumin has been reported (15, 16, 17, 18). The relative effects of bovine serum albumin and lecithin were investigated and the results obtained are given in Table I. The following general conclusions may be made from these results:

(*a*) There is an inhibitory effect of heated venom (47 μg A.P.) within ten minutes on the P/O ratio of rat liver mitochondria which takes place at

concentrations that do not inhibit respiration for a period of more than two hours.

(*b*) The effect of heated venom on oxidative phosphorylation may be reversed, either wholly or partly, by either serum albumin or egg lecithin.

(*c*) In the presence of 10 m*M* potassium fluoride, the stimulation of respiration by heated venom (1) is not observed (compare columns 1 and 4, Table I).

TABLE I

The effects of heated venom, egg lecithin, and bovine serum albumin on the P/O ratio of rat liver mitochondria

	Additions					
Egg lecithin (mg)	0	0	12	0	0	12
Bovine serum albumin (mg)	0	10	0	0	10	0
Heated A.P. venom (μg)	0	0	0	47	47	47
μmoles phosphate uptake	20.8	22.7	20.7	9.4	18.7	14.3
μatoms oxygen uptake	7.3	7.9	7.4	6.6	6.9	6.5
P/O	2.85	2.9	2.8	1.4	2.7	2.2

The conditions employed were as described under Methods and Materials. The substrate was a mixture of 6.7 m*M* pyruvate and 0.67 m*M* malate. Incubation time, over which oxygen consumption and phosphate uptake were measured, was 10 minutes.

Attempts were made to reverse this action of heated venom on the P/O ratio by addition of heated mitochondria but usually these attempts were not successful.

As the effect of heated venom on rat liver mitochondria in decreasing the P/O ratio, and increasing the rate of oxygen consumption, resembled that of 2,4-dinitrophenol (DNP) a study was made of the influence of lecithin on the action of DNP on liver mitochondrial respiration. Results given in Table II show that lecithin in contrast to serum albumin exerts no protective effect. It may, therefore, be concluded that lecithin has no ability to protect rat liver mitochondria from the uncoupling action of DNP.

TABLE II

The effects of bovine serum albumin and lecithin on the uncoupling action of 2,4-dinitrophenol

Additions			
Dinitrophenol	Lecithin	Albumin	P/O ratio
−	−	−	2.70
−	+	−	2.44
−	−	+	2.87
+	−	−	0.50
+	+	−	0.43
+	−	+	1.75

Rat kidney mitochondria were incubated in the presence of sodium pyruvate (6.7 m*M*) and potassium malate (0.67 m*M*). The additions were added as indicated, DNP (0.05 m*M*, final concentration), lecithin (8 mg/vessel), albumin (6.7 mg/vessel). Standard conditions were employed throughout.

In another series of experiments, it was found that 75 μg A.P. venom reduced the P/O ratio found with rat liver mitochondria (oxidizing pyruvate and malate) from 2.75 to 0.6. The addition of 6.4 mg lecithin increased the value to 1.9,

0.5 mg albumin increased it to 1.4, and the addition of both 6.4 mg lecithin and 0.5 mg albumin increased it to 2.55. Thus the protective effects of lecithin are seen only on the phospholipase-inhibited system and differ from those of albumin.

The results given in Table III show the effects of addition of albumin on the action of heated venom on mitochondrial respiration. There is an increase in the "fall-off" time and an absence of the initial stimulation of respiratory activity usually found on the addition of heated venom. In this respect albumin differs from lecithin. A similar result has been obtained with rat kidney mitochondria under similar conditions.

TABLE III

The effects of heated venom, egg lecithin, and bovine serum albumin on the oxidation of pyruvate by rat kidney mitochondria

	Lecithin added, mg					
	0	0	0	4.8	4.8	4.8
	Albumin added, mg					
	0	2.0	3.5	0	2.0	3.5
	μl oxygen taken up 0 – 10 min					
No heated venom added	96	90	94	90	96	94
300 μg heated A.P. venom added	122	94	94	132	92	96
	"Fall-off" time, in presence of heated venom, in min					
300 μg heated A.P. venom added	27	45	56	38	60	70

Standard manometric conditions were employed. The substrate was 6.7 m*M* pyruvate plus 0.67 m*M* malate.

In view of the protective effect of glutathione on the respiratory mechanisms, its effect on oxidative phosphorylation was also investigated. It was found that glutathione at all concentrations investigated had no effect on the P/O ratio nor on the action of venom in reducing this ratio.

The Effect of Lysolecithin on Oxidative Phosphorylation

The reports by Habermann (2) and by Witter *et al.* (14) that lysolecithin is an effective uncoupling agent might indicate that the effect of heated venom when added to respiring mitochondria is due to the formation of lysolecithin. This hypothesis was examined by comparing the effects of lecithin, heated venom, and an incubated mixture of these substances on the P/O ratio of rat liver mitochondria. The results given in Table IV were obtained. They leave no doubt that, in the absence of preincubation in an ether atmosphere, the presence of lecithin serves only to prevent the fall in the P/O ratio by the venom. After preincubation of a mixture of lecithin and heated venom in an atmosphere of ether, the mixture is a more effective uncoupling agent than the venom itself. This is presumably due to the formation of lysolecithin. However, the fact that the effect of heated venom on mitochondrial respiration takes place immediately is not compatible with the view that its effect is wholly due to the

TABLE IV

The effect of preincubation with ether on the uncoupling action of mixtures of lecithin with heated venom

Additions		Preincubation time (min)	P/O ratio
Heated R.V. venom (μg)	Lecithin (μg)		
0	0	0	2.68
0	150	0	2.56
45	0	0	1.55
45	150	0	2.22
0	0	45	2.79
0	150	45	2.69
45	0	45	2.05
45	150	45	0.94

Rat liver mitochondria were incubated under the standard conditions in the presence of 6.7 m*M* pyruvate and 0.67 m*M* malate. All values are the means obtained from at least three experiments.

formation of lysolecithin; if the latter were the case a gradually increasing effect on mitochondrial activity (e.g. on the P/O ratio) would be observed.

Structural Changes Produced by Heated Venom

It has previously been reported (3) that the addition of heated venom to a suspension of respiring mitochondria results in a marked decrease in optical density (O.D.). Witter and Cottone (19) have observed that the addition of *Crotalus adamanteous* venom to mitochondria suspended in 0.44 *M* sucrose does not lead to a decrease in O.D. whereas addition of various lysolecithins causes a marked decrease which was attributed to a swelling of the mitochondria.

Examination of the mitochondria under a phase contrast microscope indicated that there is indeed a marked destructive action of the heated venom. In certain hypotonic media, mitochondria may release into the medium an enzyme such as acid phosphatase (20). It seemed reasonable, therefore, to suppose that if heated venoms cause an impairment of the structural integrity of the mitochondria they will also cause a general release of proteins, including certain enzymes, into the medium. It has been demonstrated (21) that disruption of mitochondria leads to a release of 60% of the protein into the medium although there is a subsequent report (22) that protein may be released into the medium without disruption of the mitochondria. Cytochrome *c* is extractable from intracellular particles after treatment with phospholipase A (23, 24). It is also known that when mitochondria are aged, or when they are disrupted, there is a marked increase in the ordinarily low "latent" ATP-ase activity of the mitochondria as well as the appearance of an appreciable amount of adenosinetriphosphatase (ATP-ase) in the medium.

The influence of heated venom on the liberation of protein and ATP-ase in media containing mitochondria was investigated. Results from a typical experiment are given in Table V. There is a significant release of protein, and a simultaneous release of ATP-ase, into the medium. It should be noted that the total amount of protein in the "residual" plus "soluble" portions constituting the original suspension remains constant but the proportion of the "soluble"

TABLE V

The release of ATP-ase and total protein from rat liver mitochondria by heated venom

μg heated R.V. venom added	ATP-ase activity (μmoles P_i released in 10 minutes)		μg protein	
	Supernatant	Residue	Supernatant	Residue
0	0	5.14	170	370
3.75	0.72	7.10	265	285
7.5	1.36	7.39	300	265
14.0	1.40	7.71	360	215
45	1.70	7.16	370	200
75	1.49	6.78	380	180

Samples of mitochondria were incubated under standard conditions for 15 minutes except that histidine was used in place of phosphate as buffer. The supernatant was then separated from the residual mitochondrial substance by centrifuging and both the ATP-ase activity (35) and protein content (36) of each fraction were assayed.

is increased in presence of phospholipase A. The addition of heated venom results initially in an increase of both "supernatant" and "residual" ATP-ase activities. When more than 25 μg heated venom is added the "soluble" ATP-ase activity remains fairly constant but within the range of concentrations used there is a significant decrease in the level of ATP-ase activity associated with the residual mitochondrial particles. This fact is consistent with the claim (25) that ATP-ase is a lipoprotein which is inactivated by the alpha toxin, lecithinase C, of *Clostridium welchii*.

The Release of Enzymes from Mitochondria by Heated Venom

The demonstration that ATP-ase activity appears in the medium after incubation of mitochondria with heated venom suggested that other enzymes

TABLE VI

The effect of lecithin on the release of enzymes from rat liver mitochondria by the action of heated R.V. venom

Additions	Fractions	Succinic dehydrogenase activity (μl CO_2/15 min)	Malic dehydrogenase activity (μl CO_2/30 min)
Nil	Initial	357	209
	Supernatant	9	53
	Residue	461	140
375 μg heated R.V. venom	Initial	114	208
	Supernatant	116	188
	Residue	12	16
10 mg lecithin	Initial	386	200
	Supernatant	4	50
	Residue	463	138
10 mg lecithin plus 375 μg heated R.V. venom	Initial	123	212
	Supernatant	115	194
	Residue	6	25

Samples of mitochondria were incubated with the additions noted above for 20 minutes at 37° in a medium containing bicarbonate buffer. The various fractions were then isolated and the succinic and malic dehydrogenase activities were measured, using ferricyanide in an atmosphere of 93% N_2 – 7% CO_2 and in presence of the relevant substrates by the method of Quastel and Wheatley (37).

may also be released. The relative activities of succinic and malic dehydrogenases were estimated and the results obtained are quoted in Table VI. The observed activities of malic dehydrogenase indicate that the effect of heated venom is simply to cause the enzyme to appear in the medium — presumably by bringing about leakage from the mitochondria. The phospholipase has no effect on the total malic dehydrogenase activity and the presence of lecithin cannot prevent the release of this enzyme. Lecithin is also unable to prevent the loss of succinic dehydrogenase activity from the mitochondria nor is it able to prevent the marked loss (70%) of total succinic dehydrogenase activity, this loss being a characteristic feature of the action of phospholipase A on tissue homogenates (26, 27).

Discussion

The addition of phospholipase A, in the form of heated snake venom, to mitochondria of rat liver, kidney, or brain leads (1, 3) to an immediate increase in the rates of oxygen uptake in the presence of a variety of oxidizable substrates. Coincident with the stimulation of respiration brought about by the phospholipase is a fall in the P/O ratio which is proportional to the amount of phospholipase A added (3). The stimulation of respiration may continue long after the P/O ratio has diminished to zero, the duration of the stimulation being dependent on the amount of phospholipase A present. The more phospholipase A that is added, the sooner is the time at which respiration begins to diminish and finally ceases. The sequence of reactions undergone by mitochondria in the presence of phospholipase is, therefore, an increase in the rate of respiration together with a fall in the P/O ratio, followed by a decline and ultimate loss of respiratory activity.

At the same time there takes place a release of protein, including enzymes such as ATP-ase and malic dehydrogenase, from the mitochondria into the surrounding medium. The total ATP-ase activity of the mitochondrial system, as measured by hydrolysis of added ATP, is increased by the presence of phospholipase A. Such an increase may only be a reflection, however, of the fact that the mitochondrial membrane may be affected so that there is either an increased rate of ATP entry into the mitochondria or a liberation of ATP-ase into the medium (which definitely takes place) with a resultant increased rate of hydrolysis of the phosphate ester. Granting an immediate change in the permeability of the mitochondrial membrane on addition of phospholipase A, the increased rates of respiration may be explained by either increased rates of entry of substrates into the mitochondria or by an increased ATP-ase activity (28) or by both phenomena occurring simultaneously. The fact that the stimulation of respiration by heated venom is not observed in the presence of fluoride would favor the view that ATP-ase activity is a rate-controlling factor. If this view of the mode of action of phospholipase on mitochondria is correct, the immediate effect of the enzyme is to attack a component of the mitochondrial structure with subsequent change in membrane permeability. Such an explanation will also account for the phenomena occurring in the presence of phospholipase C.

It has been shown that on the addition of phospholipase to liver mitochondria lysolecithin is formed. It is still a matter of conjecture as to how far lysolecithin is itself directly responsible for the changes ascribed to the action of phospholipase A. Phospholipase C does not form lysolecithin and hence the problem does not arise with this enzyme.

Lysolecithin has been shown (14) to diminish the P/O ratio found with rat liver mitochondria oxidizing succinate and β-hydroxybutyrate, and to depress the rates of oxidation of these substances. Nevertheless, as Witter *et al.* (14) point out, the amounts of lysolecithin used to demonstrate these changes are equivalent to over half the amount of lecithin originally present in the mitochondria. As the fall in the P/O ratio of rat liver mitochondria takes place almost immediately after addition of phospholipase A to mitochondria, it seems unlikely that the whole of the activity is due to the formation of lysolecithin. It seems more likely that the initial fall in the P/O ratio is due to the direct effect of phospholipase A on a mitochondrial component and that the effect is enhanced by the gradual production of lysolecithin.

The losses of respiratory activity that take place in liver, kidney, and to some extent in brain mitochondria on continued exposure to phospholipases A or C are due to the complete rupture of the mitochondria with loss of components necessary for optimal respiratory activity. This conclusion is borne out by the changes in optical density of mitochondria that occur both aerobically and anaerobically in presence of phospholipase A (3). It is already known (19, 29) that ATP plays an important role in maintaining the mitochondrial structure. Loss of ATP as a result of the immediate drop in the P/O ratio together with the formation of lysolecithin from mitochondrial lecithin may partly account for the changes of the mitochondrial structure (19).

The role of lipids in electron transport is now the subject of numerous investigations (30–33) and there is little doubt that the destructive effect of phospholipase A or C on tissue respiration (26, 27, 34) is due to the removal of lipids essential both for oxidative phosphorylations and for the integrity of mitochondrial structure.

Lecithin protects rat liver mitochondria from the inhibitory action of phospholipase A and even the presence of heated mitochondria has a similar protective effect. Whether this protection is due to competition by the lecithin for the enzyme, or whether it is due to a direct interaction between the phospholipid and the mitochondria, is as yet undecided. No protection is afforded against the inhibitory action of phospholipase C. Both lecithin and serum albumin diminish the fall of the P/O ratio by phospholipase A, but serum albumin protects mitochondria from the effects of a variety of uncoupling agents such as dinitrophenol. The effect of lecithin appears to be specifically connected with the uncoupling action of phospholipase A. Serum albumin, however, in contrast to lecithin brings about a decrease of the initial stimulated rate of respiration of mitochondria in presence of phospholipase A to the unstimulated rate. Possibly albumin protects mitochondrial structure by combining with components of the mitochondria and preventing the interaction

between phospholipase and mitochondria, for it also prolongs the time before the eventual decline of the respiratory rate takes place (Table III).

It is concluded that the first effects of the venom phospholipase consist of a release of protein (including ATP-ase and other enzymes) from the mitochondria together with a fall in the P/O ratio. Both these effects may be considered as a result of the attack on phospholipids in the mitochondrial structure. The initial increases of rates of respiration are most likely due to consequent changes such as an increase in mitochondrial permeability or the observed increase of ATP-ase activity of the system. The phenomena of the stimulated rate of oxygen consumption, the fall in P/O ratio, and increased ATP-ase activity all seem to be reflections of initial changes in the mitochondria due to the initial attack by phospholipase A, with change or removal of essential phospholipids. Hydrolysis of these components eventually eliminates phosphorylation without necessarily disturbing respiratory activity. The phospholipase may then hydrolyze other phospholipids which are either constituent parts of the respiratory enzymes or which act as a medium that binds these enzymes together in the particles and thus allows electron transport to take place. When a sufficient amount of phospholipid has been removed, the spatial configurations of the remaining constituents of the particles are disturbed with ultimate rupture of the mitochondria, there is a marked decrease in optical density and respiratory activity ceases.

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ACID-LABILE PHOSPHATE CONTENT OF INCUBATED BRAIN SLICES[1]

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Abstract

During aerobic incubation of brain cortex slices in glucose-containing medium at 38° the pyrophosphate content of the tissue increases rapidly for 15 minutes and then more slowly. The creatine phosphate content increases rapidly during the first 5 minutes and then falls. Anaerobically, the pyrophosphate falls during the first 30 minutes; the creatine phosphate tends to rise initially and then falls to a lower level than that found aerobically. In the absence of glucose and oxygen the levels of both substances fall rapidly to low levels but can be raised again by aerobiosis in the presence of glucose. In the absence of glucose and oxygen in ice-cold medium, or at 38° in the absence of any medium, the levels of pyrophosphate and creatine phosphate are preserved. More inorganic phosphate is produced when slices are incubated than can be accounted for by breakdown of acid-labile phosphate compounds.

It is shown that the initial rate of anaerobic glycolysis by slices which have been subjected to various pretreatments is strongly correlated with the pyrophosphate content of the tissue.

The failure of slices to concentrate potassium when immersed in sodium-free media is not due to failure to maintain pyrophosphate levels.

Introduction

The present study was undertaken to illuminate two different sets of observations.

Elliott and Rosenfeld (1) showed that brain slices, which had lost their ability to carry on anaerobic glycolysis as a result of a period of deprivation of oxygen and glucose at 38°, could be reactivated by supplying the tissue with oxygen for a brief period. They also showed that deprivation of oxygen and glucose did not cause loss of glycolytic ability if the slices were incubated without medium or in ice-cold medium. In view of the well-known facts that adenosine triphosphate (ATP) is necessary for the initiation of glycolysis and is liberally produced during aerobic metabolism, the possibility has been examined that anaerobic glycolytic activity, its restoration, and its preservation, might be correlated with the availability of acid-labile phosphate. Some years ago Macfarlane and Weil-Malherbe (2) found that the loss of glycolytic activity by rabbit brain slices after anaerobic incubation without glucose was accompanied by a decrease in pyrophosphate content of the slices. The results now obtained indicate that the variations in glycolytic activity which follow various pretreatments of the slices are well correlated with the level of pyrophosphate in the tissue.

Pappius, Rosenfeld, Johnson, and Elliott (3) showed that the ability of metabolizing brain slices to maintain a high concentration of potassium depends upon the presence of sodium in the surrounding medium. This could

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be explained by the activity of a mechanism which extrudes sodium specifically. However, the earlier results did not exclude the possibility that the accumulation of potassium in slices is dependent on the presence of adequate concentrations of energy-rich phosphate and that sodium is specifically necessary for the maintenance of the requisite level of such phosphate compounds. Accordingly the level of acid-labile phosphate compounds in brain slices incubated in sodium-free media has been determined. The results obtained indicate that the requirement for sodium in the medium is independent of the provision of energy-rich phosphate groups in the slice.

In the course of this investigation information was obtained about the time course of changes in the pyrophosphate-P[2] (Pyro-P) and creatine phosphate (CrP) contents of rat brain slices under various conditions in which oxygen consumption, glycolysis, and potassium content have previously been studied. This may be of more general interest.

Methods

General Procedure

Slices of rat cerebral cortex were prepared in a humid chamber, weighed (usually 70–110 mg), and incubated as previously described (Pappius and Elliott (4)). For incubation without medium, slices were placed in dry Warburg vessels containing wet filter paper in the center well to maintain humidity.

The media used were the "normal" glucose-containing bicarbonate-buffered saline medium (4), the same medium lacking glucose, and the sodium-free media described by Pappius *et al.* (3). The gas phase was either oxygen– or nitrogen – 5% carbon dioxide.

At the end of an incubation the vessel was disconnected from the manometer and covered with a perforated porcelain disk, while the relevant gas was passed into the vessel through the side arm. Then the vessel was tipped so that the medium passed through the disk and the slices were retained. The disk and tissue were dropped immediately into small beakers containing the appropriate reagents. The disk was removed and the tissue and reagents were transferred to a homogenizing tube and immediately homogenized. This procedure of gassing with the appropriate gas during the separation of tissue from the medium was necessary after anaerobic incubation, since even brief exposure to oxygen tended to raise the level of high-energy phosphates in the tissue. For the sake of uniformity this technique was applied for all experimental conditions. Unincubated tissue was dropped directly into the homogenizing tube containing the reagents; tissue incubated in the absence of medium was rinsed with the reagents from the vessel into the tube. Four identically set up vessels and slices were used for each complete phosphate determination—two for duplicate estimations of the inorganic phosphate and two for duplicate determinations of the other phosphate fractions.

[2]The pyrophosphate-P includes mainly the pyrophosphate groups of adenosine tri- and di-phosphate (ATP and ADP). Presumably other nucleotide-phosphate compounds are also included in the determinations.

Determination of Tissue Phosphates

Inorganic Phosphate (P_i)

The tissue sample was homogenized in 2.9 ml of the cold sulphate–acetate reagent of Lowry and Lopez (5) and the homogenate was filtered and kept in the refrigerator until the other samples were ready. Inorganic phosphate was determined on 0.5-ml or 1-ml aliquots of the filtrate by the method of Lowry and Lopez with the addition of 5×10^{-5} *M* $CuSO_4.5H_2O$ as suggested by Peel and Loughman (6). The first reading, taken 10 minutes after addition of the molybdate reagent, was not significantly different from a further reading taken 10 minutes later, as suggested by Lowry and Lopez, which indicated that no hydrolysis of phosphocreatine occurred.

Acid-Labile Organic Phosphates

The tissue samples were homogenized in 7.9 ml of 7% trichloracetic acid and the homogenates were filtered. An aliquot, 1 to 3 ml, of the filtrate was made up to 3 ml with 7% trichloracetic acid and 0.3 ml of concentrated hydrochloric acid was added. One of the aliquots was heated in a boiling-water bath for 8 minutes and cooled, and its phosphate content was determined by the method of Fiske and SubbaRow (see ref. 7). The phosphate fraction, thus determined, which we designate P_h, represents mainly the inorganic phosphate plus phosphate derived from adenosine tri- and di-phosphates and creatine phosphate.[3] In the present experiments the hydrolysis of known amounts of ATP was 96% effective (64% of total phosphate liberated instead of the expected 67%).

The phosphate content of another HCl-acidified aliquot of the trichloracetic acid extract was determined after standing for 15 to 20 minutes at room temperature, also by the method of Fiske and SubbaRow. The phosphate fraction thus determined is designated P_i–TCA and represents the inorganic phosphate plus the easily hydrolyzed phosphate of creatine phosphate. In the present experiments hydrolysis of ATP under these conditions was less than 6%.

The values reported for the pyrophosphate P (Pyro-P, mainly from ATP and ADP) are the differences between the values obtained for P_h and P_i–TCA on aliquots of the same filtrate. The values for creatine phosphate (CrP) are the differences between the means of duplicate values of P_i–TCA and P_i for individual experiments.

The inorganic phosphate content of slices is quite high and the values obtained for Pyro-P and CrP are based on rather small differences between P_h and P_i–TCA or between P_i–TCA and P_i. It was necessary to pay close attention to details such as the uniformity of colorimeter tubes in order to obtain reasonably consistent results and there was considerable variability in the results of different experiments under the same conditions. In some

[3]In some early experiments the fraction P_h was determined by the method of Lowry and Lopez (5). The HCl-acidified, heated trichloracetic acid filtrate was neutralized to pH 4 with sodium acetate and the phosphate then determined in the same way as for P_i. In a number of comparisons the P_h values obtained by the two methods were the same. This method for hydrolyzed samples was, however, abandoned since in occasional experiments the standard curves were not linear and the results had to be discarded.

experiments in which low Pyro-P values were expected the weight of tissue slices used was doubled. The standard errors of the mean results are indicated in the tables and figures.

Other Determinations

Rates of anaerobic glycolysis were determined by standard Warburg manometric procedure at 38°, usually after previous incubation under various conditions. Results shown in Fig. 2 give rates during the first 20 minutes after pretreatment; those in Table II represent the rate over the last 10 minutes of anaerobic incubation.

Potassium was determined by flame photometry after digestion of the slices with nitric acid and addition of excess sodium according to Pappius and Elliott (8).

Results

Changes in Pyrophosphate and Creatine Phosphate with Time

Aerobic

Fig. 1*a* shows the changes in the content of Pyro-P and of CrP of slices incubated in the usual Ringer–bicarbonate–glucose medium in the presence of oxygen and carbon dioxide. In setting up the experiments, slices stood for 5–10 minutes in the saline medium at room temperature while other slices were being weighed and the vessels were being gassed. During this "setting-up" period the Pyro-P and CrP contents of the slices did not change significantly. On incubation at 38° the Pyro-P rose rapidly for 15 minutes and then more slowly. The CrP rose sharply during the first 5 minutes of incubation to a level considerably above the value for fresh tissue. Thereafter the CrP concentration fell until, at 30 minutes, a level was reached which was subsequently maintained.

As previously observed by Thomas (9) the presence of 1 mM of added creatine in the medium caused an appreciable increase in the CrP content of the tissue after 120 minutes' incubation. The Pyro-P appeared also to increase somewhat. No appreciable effects were noted after 60 minutes' incubation.

Anaerobic

Under anaerobic conditions with glucose present (Fig. 1*b*) the Pyro-P content fell to about half of its original value during the first 20–30 minutes and then remained fairly constant. The CrP content tended to fall during the setting-up period, to rise again during the first 10 minutes at 38°, and then to fall to a value lower than that found under aerobic conditions. (The average for all values for CrP at 10 and 20 minutes anaerobiosis is significantly higher ($P<0.05$) than the average of the values at all other times. The average values after 60 minutes' aerobic and anaerobic incubation with glucose also differed significantly ($P<0.05$).)

Stimulation of the anaerobic metabolism by a preliminary period of aerobiosis and the addition of pyruvate brought about an increase ($P<0.02$) in the Pyro-P content over that found after direct anaerobic incubation but did not raise the CrP level.

Anaerobic in the Absence of Glucose, and Related Conditions

In the absence of oxygen and of glucose in the medium the Pyro-P content of the tissue fell to about a third of the initial value during the first 5 minutes and remained at this level (Fig. 1*c*). CrP also fell to a low value but more slowly.

When, after 30 minutes anaerobic incubation in glucose-free medium, glucose was added and conditions were made aerobic for 20 minutes (Fig. 1*c*), the content of Pyro-P rose by approximately as much as the initial rise obtained with oxygen and glucose immediately available. However, the level

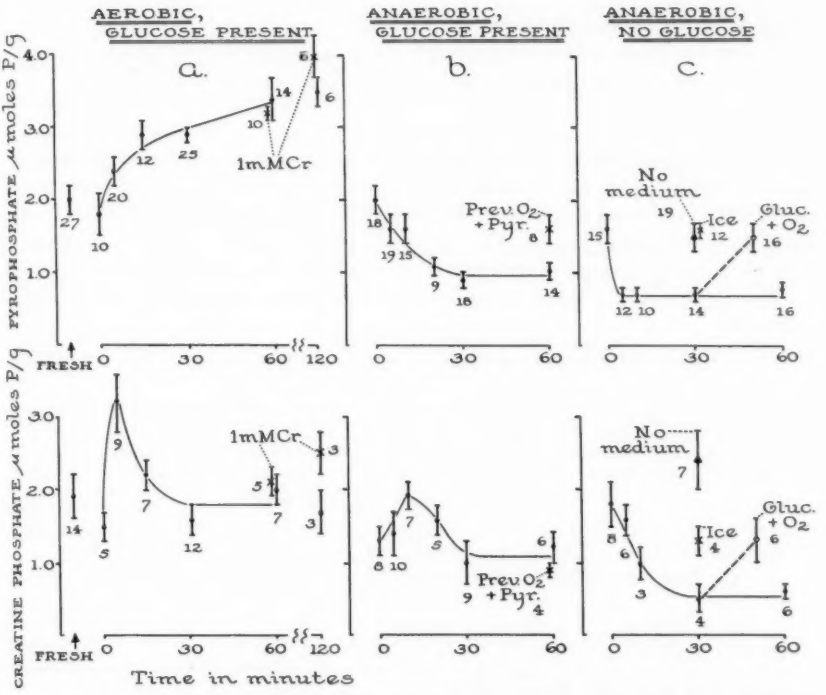

FIG. 1. Changes in pyrophosphate and creatine phosphate content of cerebral cortex slices during incubation at 38° C.

Averages ± standard error. The number next to each point is the number of determinations.

0 time. Tissue which has been in the medium, during preparation of vessels and gassing for 10 minutes or less at room temperature.

(*a*) Slices incubated aerobically in glucose-containing medium for various periods, and in medium containing 1 m*M* creatine for 60 minutes and 120 minutes.

(*b*) Slices incubated anaerobically in glucose-containing medium for various periods. For the points marked "Prev O_2 + Pyr" the tissue was incubated aerobically for 25 minutes, then 2 m*M* pyruvate was added to the medium and anaerobiosis was established and maintained for 60 minutes.

(*c*) Slices incubated anaerobically without glucose. "Ice": incubation in ice-cold medium. "No medium": incubation without medium in moist nitrogen – 5% carbon dioxide. "Gluc + O_2": after 30 minutes of anaerobic incubation in medium without glucose, glucose was added and the gas phase changed to oxygen – 5% carbon dioxide.

reached was considerably lower than in tissue which had never been deprived of oxygen and glucose. The level of CrP also rose, but did not reach the steadily maintained aerobic level.

When the tissue was incubated anaerobically without glucose for 30 minutes in ice-cold medium instead of at 38° the Pyro-P content did not fall significantly and the decrease of the CrP was greatly diminished.

The energy-rich phosphate compounds were also preserved during anaerobic incubation of the tissue at 38° if the tissue was not immersed in the saline medium. Neither the Pyro-P nor the CrP content were significantly changed from the preincubation level (Fig. 1*c*).

Total Acid-Labile and Inorganic Phosphate

Values for total acid-labile phosphate plus inorganic phosphate (fraction P_h) and inorganic phosphate (fraction P_i) are summarized in Table I.

TABLE I

Total acid-labile plus inorganic phosphate and inorganic phosphate, after incubation

Incubation time, min	μmoles P per g fresh tissue					
	Aerobic with glucose		Anaerobic with glucose		Anaerobic no glucose	
	P_h	P_i	P_h	P_i	P_h	P_i
No medium						
0	(Fresh unincubated tissue slices)				19.0 (47)	15.5 (46)
30	(Incubated 30 min anaerobically without medium)				23.1*(23)	19.4*(20)
In medium	P in the tissue					
0†	15.0*(16)	12.1*(16)	15.9 (18)	12.7 (19)	16.7*(16)	13.4 (16)
5	13.1 (20)	7.9 (20)	13.6 (19)	10.5 (22)	13.6*(14)	11.5 (16)
30	9.2 (54)	4.9 (49)	9.6 (33)	7.6 (29)	9.8 (34)	8.0 (28)
60	9.1 (24)	4.1 (23)	8.1 (16)	6.5 (14)	9.3 (16)	7.9 (12)
	P in the medium after 30 min incubation					
	11.7 (12) ±0.6	13.0 (11) ±0.4	14.3 (6) ±0.6	15.3 (6) ±0.6	12.7 (6) ±1.1	13.4 (6) ±1.2
	Excess in tissue plus medium after 30 min incubation over that in fresh unincubated tissue					
	1.9	2.4	4.9	7.4	3.5	5.9

NOTE: Averages. Numbers of determinations in parentheses. The standard error was ±0.3 μmoles P/g or less except in cases marked with * where it was 0.4 or 0.5.
†In medium for 5–10 minutes at room temperature during setting up.
P_h = total acid-labile plus inorganic phosphate. P_i = inorganic phosphate.

In tissue which had been incubated in the absence of medium the P_h and P_i were increased to well above the levels present in fresh tissue. During the first few minutes after immersion of slices in saline medium there was a considerable decrease in the P_h and P_i of the tissue. Little further loss from the tissue occurred after 30 minutes of incubation. Of the phosphate remaining in the tissue a greater proportion was found in inorganic form when metabolism was depressed by lack of oxygen and/or glucose.

Phosphate determinations on the media in which slices had been incubated showed that more phosphate was present in the medium than could be ac-

counted for by loss of acid-labile phosphate or inorganic phosphate from the tissue. The figures for media given in Table I are based on rather few, variable, determinations but it appears[4] that the excess phosphate appearing is inorganic phosphate and a greater excess is found under anaerobic than under aerobic conditions.

Glycolysis and High-Energy Phosphate Levels after Pretreatments of Tissue

In Fig. 2 the levels of Pyro-P and of CrP found in brain cortex slices after a number of treatments are plotted against the rates of anaerobic glycolysis found immediately after such treatments. (Most of the rates shown were determined in previous work (1, 3).) The initial rate of glycolysis was strongly correlated with the level of Pyro-P found in tissue subjected to the same antecedent conditions ($r=0.98$, $n=9$, $P<0.01$). The correlation with the CrP level was not significant ($r=0.58$, $n=9$, $P>0.05$).

Pyrophosphate and Creatine Phosphate Content of Tissue Incubated in Sodium-Free Media

The high-energy phosphate contents of brain slices after incubation under various conditions in the usual bicarbonate–glucose–saline medium and in three types of sodium-free media (sodium replaced by potassium, lithium, or choline) are shown in Table II. Included in Table II are figures for glycolytic activity and potassium content obtained previously (Pappius and Elliott (8), Pappius *et al.* (3)) under these experimental conditions.[5]

TABLE II

Acid-labile phosphate, final glycolysis rates, and potassium contents of slices of rat cerebral cortex incubated at 38° C

Experimental conditions	Main cation in medium	Pyro-P (μmoles P/g fresh weight)	CrP (μmoles P/g fresh weight)	Glycolysis ml CO_2/g/hr, *last* 10 min	Potassium mmoles/kg fresh weight
Unincubated tissue		2.0 (27)	1.9 (14)		109 (14)
Aerobic—60 min	Sodium	3.4* (14)	2.0 (7)		55 (5)
	Potassium	2.5 (8)	1.2 (4)		284 (3)
	Lithium	2.45*(8)	0.9 (4)		20 (5)
	Choline	2.8 (6)	1.3 (3)		21 (4)
Anaerobic—60 min	Sodium	1.0 (14)	1.2 (6)	0.7 (26)	19 (14)
	Potassium	1.3 (6)	0.7 (3)	1.25 (12)	271 (4)
	Lithium	0.9 (10)	1.4 (5)	0.5 (10)	16 (4)
	Choline	1.55 (8)	0.7 (4)	1.95 (16)	16 (12)
Anaerobic—60 min with previous aerobiosis (25 min) and pyruvate (2 m*M*)	Sodium	1.6 (8)	0.9 (4)	2.5 (20)	32 (14)
	Potassium	1.5 (8)	1.3 (4)	2.0 (6)	307 (4)
	Lithium	1.0 (8)	1.2 (4)	1.3 (10)	15 (4)
	Choline	1.8 (10)	1.0 (5)	3.3 (10)	15 (11)

NOTE: Averages. Number of determinations in parentheses.
The potassium and glycolysis results are taken from Tables I (corrected see footnote [5]) and III of Pappius *et al.* (3), together with additional results obtained in the present investigation for the choline medium. The figure for potassium in unincubated tissue is calculated from results in Pappius and Elliott (8) Table V.
The standard error of the mean for Pyro-P was ±0.2 μmole P/g or less, except in two cases marked * where it was ±0.3 μmole P/g. For CrP it was ±0.3 μmole P/g or less.

[4]In Table I the figures for media have been related to the average of all relevant determinations. The same results were definitely apparent, and of similar magnitude, from determinations on unincubated slices and incubated slices and media in individual experiments.

[5]In Table I of the paper by Pappius *et al.* (3) the figures for potassium content after incubation in lithium and tris media are interchanged. This error makes no difference to the conclusions in that paper.

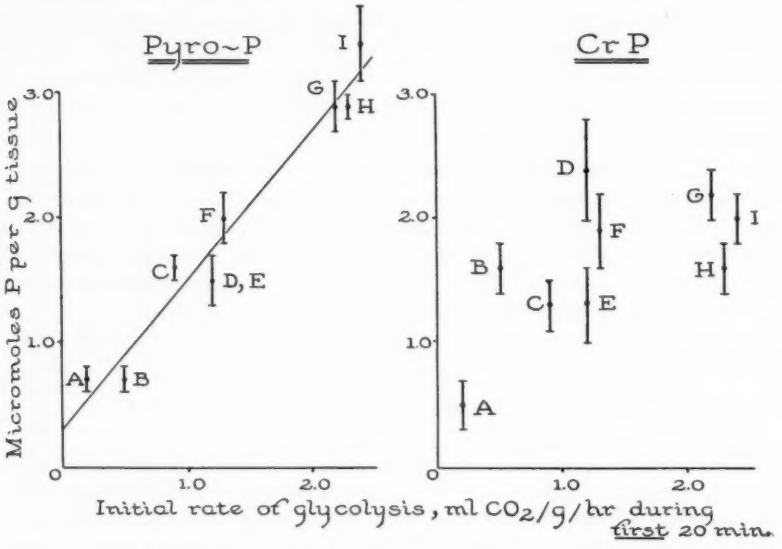

FIG. 2. Initial rates of anaerobic glycolysis vs. acid-labile phosphate levels in cerebral cortex slices after various pretreatments.

PRETREATMENTS

A. Anaerobic, no glucose, 30 minutes, 38° C
B. Anaerobic, no glucose, 5 minutes, 38° C
C. Anaerobic, no glucose, ice-cold medium 30 minutes.
D. Anaerobic, no medium, 30 minutes, 38° C
E. Anaerobic, no glucose, 30 minutes, 38° C then glucose added and aerobic 20 minutes, 38° C
F. No deliberate pretreatment (fresh tissue)
G. Aerobic, glucose present, 15 minutes, 38° C
H. Aerobic, glucose present, 30 minutes, 38° C
I. Aerobic, glucose present, 60 minutes, 38° C

Rates of glycolysis for pretreatments 1–5 are averages from Elliott and Rosenfeld (1), for pretreatment 6 from Pappius *et al.* (3), for pretreatments 7, 8, and 9 from present work (averages of 6, 26, and 4 determinations respectively).

Pyrophosphate-P averages. Vertical lines indicate standard error. Twelve to twenty-seven determinations for each pretreatment. Regression line calculated from averages.

Creatine phosphate averages. Vertical lines indicate standard error. Four to fourteen determinations for each pretreatment.

Under aerobic conditions with sodium-free media the Pyro-P level increased above that in unincubated tissue though the level reached was significantly lower than in the sodium-containing medium. The CrP level fell considerably lower than in the sodium-containing medium.

Under anaerobic conditions, direct or after treatment which stimulates glycolysis, the Pyro-P content of the slices in the various media varied with the rate of glycolysis. This is discussed later. The CrP level varied in a manner for which we have no explanation.

Discussion

Relation of Present Results to Those of Other Workers

The values for Pyro-P and Cr-P reported in the present work for fresh tissue are lower than most of those reported for tissue frozen *in situ* (see 10, 11, 12). Immediate chemical immobilization is necessary to determine the levels of these compounds in vivo. In our experience, however, there was no further rapid decrease in these substances when whole brain or cerebral cortex slices, not immersed in saline solution, were left at room temperature in a humid chamber for 30 minutes, or even at 38° C in the absence of oxygen (see Fig. 1*c*).

Our figures for the CrP content of slices before incubation agree closely with figures given by McIlwain *et al.* (11) for guinea pig brain slices cut "dry". McIlwain *et al.* (11) found rapid loss of phosphocreatine when the tissue was kept in saline solutions at room temperature prior to metabolic studies but, after incubation, they and others in the same laboratory (9, 13, 14) found that the CrP in the slices had risen again. Our results with rat brain incubated under similar conditions are very similar to theirs, or somewhat higher. Our figures would be expected to be higher since they are based on the fresh weight of tissue whereas the others are based on the weight of tissue swollen after immersion in saline medium. The previous workers apparently did not measure CrP early in incubation so the striking initial rise in CrP has not been previously reported.

In guinea-pig cortex slices, unincubated and incubated 60 minutes with glucose and oxygen, Kratzning and Narayanaswami (13) found 1.46 and 2.02 μmoles ATP-P/g respectively, by enzymatic determination, and 1.79 and 2.18 μmoles Pyro-P by chemical determination after calcium–ethanol separation. In tissue incubated for 30 minutes, Heald (14) found 1.4 μmoles/g of ATP-P by chemical determination after barium fractionation. Our figures for the Pyro-P content of rat cortex slices, 2.0 and 3.4 μmoles/g for fresh tissue and for slices incubated 60 minutes aerobically in ordinary medium, are higher than those just mentioned. As in the case of CrP the difference can be partly explained by the weight basis used. Part of the difference would be due to losses from the tissue when the slices are prepared wet and stored in saline before incubation as was done by the previous workers. But much of the difference is no doubt due to the fact that their methods were more specific than ours. The method used in the present study does not distinguish between the pyrophosphate P of ATP and ADP. In brain frozen *in situ* in normal animals the amount of ADP appears to be a small proportion of the total adenylpolyphosphate (12, 13, 15, 16). Under conditions of ischemia and anoxia the proportion of ADP increased very considerably (12). In incubated brain slices Kratzning and Narayanaswami (13) found no significant amounts of ADP in slices respiring in glucose-containing medium but some ADP was found when the maintenance of the high-energy phosphate level was interfered with by the presence of dinitrophenol. In the present experiments it is probable that under aerobic conditions most of the Pyro-P determined re-

presents ATP but under anaerobic conditions ADP may account for part of the Pyro-P determined.

The previous workers did not follow the time course of ATP changes.

Relation between CrP and Pyro-P

Though active metabolism might be expected to produce high levels of both CrP and of Pyro-P, Kratzning and Narayanaswami (13) concluded that their results with slices did not support the idea that there is a direct relation between changes in CrP and Pyro-P. Figure 1 shows that changes in CrP and Pyro-P are not always even in the same direction. Under aerobic conditions CrP reaches a maximum level several minutes before Pyro-P does and, at 15 minutes, the level of CrP is falling rapidly when the Pyro-P level is reaching its maximum. Anaerobically with glucose, Pyro-P falls steadily for 30 minutes but CrP rises for 10 minutes and then falls. When glycolysis is stimulated by previous aerobiosis and added pyruvate, the final level of Pyro-P, but not of CrP, is increased. Anaerobically without glucose, Pyro-P and CrP change in the same direction but Pyro-P seemed to fall more rapidly than CrP.[6]

Most of these results may be explained by the observation of Thomas (9) that the creatine content of tissue decreases during incubation. In the early stages of incubation the creatine present in the tissue could accept phosphate readily, presumably from ATP. But the free creatine which is in equilibrium with CrP is gradually lost so that less total creatine is present after incubation and therefore less CrP can be maintained. Thomas showed that, in the presence of oxygen and glucose, the tissue can take up creatine which has been added to the suspending medium. As expected from this, the CrP content of incubated tissue can be raised by adding creatine to the medium (Thomas (9), see also Fig. 1*a*). When tissue is incubated without medium, creatine cannot diffuse away and the CrP content of the tissue is maintained.

Loss from Tissue, and Production of Extra, Inorganic Phosphate

The fact that phosphate is lost from the tissue into the medium early in incubation with little subsequent loss may be accounted for in two ways. (*a*) The phosphate may be present in two conditions or compartments of the tissue from only one of which it can diffuse out readily. (*b*) Inorganic phosphate diffuses out continuously; the rate is rapid at first because the amount in the tissue is high following the initial manipulations. Thereafter the loss of inorganic phosphate from the tissue is nearly balanced by production of free phosphate from compounds which are not labile in fresh tissue.

McIlwain *et al.* (11) showed that the medium in which guinea-pig brain slices were incubated gained some inorganic phosphate from compounds other than CrP. The present experiments show that extra inorganic phosphate appears when slices are incubated, with or without medium, and this phosphate is not derived from the total acid-labile phosphate. Its source is not known.

[6]This observation was surprising since Thorn *et al.* (12) found that, in brain frozen *in situ*, the CrP falls during anoxia or ischemia more rapidly than does the ATP.

Relation of the Pyrophosphate Level to Rate of Glycolysis

The regression line for Pyro-P against rate of anaerobic glycolysis shown in Fig. 2 suggests that no glycolysis should take place if the Pyro-P level falls below 0.3 μmoles P/g. Brain slices incubated anaerobically in the absence of glucose for 30 minutes are unable to glycolyze glucose subsequently added (1, 17). It will be seen in Fig. 2 (pretreatment A) that, under such conditions, the level of this phosphate fraction is close to this critical level. Elliott and Rosenfeld (1) reported that if their slices were kept in ice-cold medium instead of at 38° C during the pretreatment, or if the slices were incubated without suspending medium, subsequent glycolysis was unimpaired. Under either of these conditions it was found, in the present experiments (Fig. 2 pretreatments C and D), that the Pyro-P is fairly well preserved. The inhibition of glycolysis by previous deprivation of oxygen and glucose at 38° can be largely reversed, by a period of aerobiosis (1). This treatment (pretreatment E) restores the level of Pyro-P to nearly the same level as in untreated tissue ("pretreatment" F). High rates of anaerobic glycolysis are obtained with tissue which has not suffered a period of deprivation but has been subjected to aerobic conditions and, in such tissue, a high level of Pyro-P is found (pretreatments G, H, and I).

The effects on glycolysis of the various pretreatments of the tissue are therefore correlated with effects on the pyrophosphate-P level. The Pyro-P level presumably reflects the ATP level; it is almost certainly the latter which determines the initial rate of glycolysis in brain slices.

Changes in rate of anaerobic glycolysis with time after its initiation are less closely related to the Pyro-P level. Although the rate of glycolysis falls rather steadily, but not rapidly, with time (see for example, figures in (1)) the Pyro-P level falls rapidly during the first 30 minutes and then remains fairly steady (Fig. 1*b*). The relatively steady level of Pyro-P found after 30 to 60 minutes incubation does, however, seem to be related to the rate at which glycolysis has just been progressing and it is probably largely *determined* by this rate. The Pyro-P levels and the *final rates* of glycolysis after 60 minutes' incubation are well correlated ($r=0.95$, $n=9$, $P<0.01$). This correlation was calculated from the average values given in Table II for the glycolysis and Pyro-P content *after* 60 minutes anaerobiosis in normal and sodium-free media, with and without previous aerobiosis plus pyruvate, together with the results obtained after 60 minutes' anaerobiosis in glucose-free, ordinary medium (Fig. 1*c*).

Relation of High-Energy Phosphate to Potassium Accumulation

The failure to accumulate potassium when tissue is immersed in sodium-free media cannot be ascribed to an effect of lack of sodium on the formation or maintenance of Pyro-P in the tissue.

The Pyro-P content of tissue incubated aerobically in the lithium or choline medium, when potassium accumulation does not occur, is higher than the Pyro-P content of tissue incubated anaerobically in the sodium-containing medium, with glycolysis stimulated by previous aerobiosis and pyruvate,

when considerable potassium accumulation occurs. Under anaerobic conditions with stimulated glycolysis, the Pyro-P content was slightly higher with the choline medium, when potassium accumulation did not occur, than in the sodium medium when accumulation did occur.

It thus appears that the ability of slices to maintain a high concentration of potassium depends directly on the presence of sodium though high-energy phosphate is undoubtedly required.

Acknowledgment

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THE FORMATION OF 6-MERCAPTOPURINE RIBOSIDE PHOSPHATE IN ASCITES TUMOR CELLS[1]

A. R. P. PATERSON

Abstract

A nucleotide metabolite of 6-mercaptopurine has been isolated from Erhlich ascites carcinoma cells exposed to this compound under in vivo and in vitro conditions. By chemical and enzymatic methods, this nucleotide has been identified as 6-mercaptopurine nucleoside-5'-monophosphate.

6-Mercaptopurine nucleotide is formed rapidly in the tumor cells in vivo, maximum concentrations being achieved within 0.5 hours after administration of the analogue. Treatment of the tumor cells with 6-mercaptopurine or with azaserine induced a twofold to threefold enhancement in their ability to synthesize 6-mercaptopurine nucleotide.

Using isotopic techniques small amounts of 6-mercaptopurine nucleotide were detected in liver, intestine, and a solid tumor. The conversion of 6-mercaptopurine to the nucleotide form was also demonstrated in an ascitic form of Sarcoma 180.

Introduction

Recently it has been recognized that 6-mercaptopurine (6-MP) (1) and several other purine analogues possessing important pharmacological properties may be converted enzymatically to nucleotide derivatives. Nucleotide formation has been demonstrated to occur with 2,6-diaminopurine (2), 8-azaguanine (3), purine (4), thioguanine (5), and the adenine, guanine, and hypoxanthine analogues of the pyrazolo (3,4-*d*) pyrimidine series (3). The formation of nucleoside and nucleotide derivatives has also been established for a number of pyrimidine analogues (see reviews by Skipper and Bennett (6), and Shive and Skinner (7)).

The present report is concerned with experiments in which it was recognized that Ehrlich ascites carcinoma cells, when treated in vivo with 6-MP, or incubated with this compound in vitro, form 6-mercaptopurine riboside-5'-monophosphate (6-MP nucleotide). The isolation and identification of this nucleotide are described and certain of its spectral properties are defined. Some of the factors which influence the formation of this compound have been evaluated and it has been concluded that the formation of 6-MP nucleotide is a major metabolic fate of 6-MP in the tumor cells.

Methods

The Ehrlich ascites carcinoma (obtained originally from Dr. C. C. Stock of the Sloan-Kettering Institute for Cancer Research) is maintained in this laboratory by weekly transplantation in female Swiss albino mice from the colony of the University of British Columbia, using inocula of 5.5×10^6 cells.

[1]Manuscript received March 21, 1959.
Contribution from the Department of Biochemistry, University of Brit sh Columbia, Vancouver 8, British Columbia.

In the following experiments the metabolism of 6-MP was studied by ion-exchange chromatography of the acid-soluble fraction of ascites tumor cells which were exposed to the analogue in vivo. Seven or eight days after transplantation with the ascites tumor, mice were injected intraperitoneally with a test dose of 6-MP dissolved in isotonic saline.[2] The dose of 6-MP ordinarily administered was 5 μmoles per animal, with adjustments made for the distention of some animals to give a dosage level of approximately 4 μmoles of 6-MP per ml of packed tumor cells. At a definite time interval after the injection of 6-MP, the mice were killed by cervical dislocation, the abdominal skin was reflected, the ascitic fluid drained through an incision into chilled, heparinized centrifuge tubes, and the tumor cells were collected by centrifuging at $1500 \times g$ for 5 minutes.

Acid-soluble fractions of the tumor cells were prepared by suspending 10–20 ml of packed tumor cells in two volumes of cold, 0.4 *M* perchloric acid. After allowing the preparation to stand in cracked ice for 15 minutes, the acid-insoluble residue was sedimented by centrifuging and the extract reserved. The residue was extracted again with 1.5 volumes of cold 0.2 *M* perchloric acid and the two extracts were combined, neutralized with potassium hydroxide, and stored at $-10°$ C. Prior to use, the frozen extracts were thawed at room temperature.

The neutralized extracts were centrifuged free of potassium perchlorate and were chromatographed on 1×20 cm columns of Dowex-1-formate anion exchange resin, using the formic acid gradient elution system described in Fig. 1. The gradient elution apparatus delivered to the column a formic acid solution, the concentration of which increased in a linear fashion with respect to the volume delivered. Column eluates were collected in 5.0-ml increments with an automatic fraction collector. The eluate fractions were examined spectrophotometrically at 260 mμ to locate the naturally occurring purine and pyrimidine nucleotides and at 323 mμ to locate 6-MP and its nucleotide.

Results and Discussion

The Identification of 6-MP Nucleotide

Figure 1 represents a typical chromatogram of the acid-soluble fraction of Ehrlich ascites cells collected 0.5 hour after the administration of 6-MP. The open profile indicates that there are two principal peaks of absorption at 323 mμ. The first peak was recognized as free 6-MP by its position on the ion-exchange chromatogram and by paper chromatography. The second peak of 323 mμ absorption, which followed adenosine diphosphate, was found to be a 6-MP-containing nucleotide, probably 6-MP riboside-5′-monophosphate.

For the chemical analyses and enzyme experiments described below, 6-MP nucleotide was prepared by reserving the evaporated residues from the 6-MP nucleotide fractions of various chromatograms until the accumulation was

[2]The test dose was administered as a solution containing 10 μmoles of 6-MP per ml in isotonic sodium chloride. This concentration, which exceeds the solubility of 6-MP at room temperature was achieved by heating the solution. The solution was cooled then to about 40° C before use and was injected before any precipitation occurred.

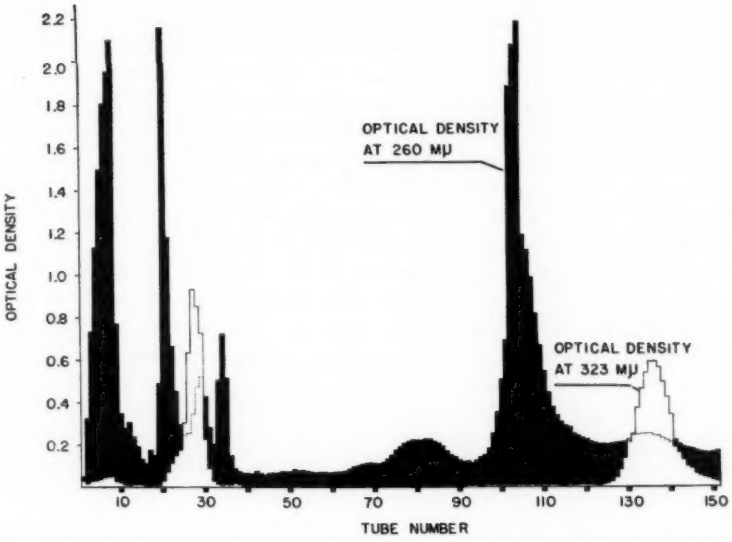

FIG. 1. Ion-exchange chromatogram of the soluble nucleotide fraction of Ehrlich ascites carcinoma cells treated in vivo with 6-mercaptopurine.

Seven days after tumor transplantation, nine mice were injected with a total of 52 μmoles of 6-MP dissolved in isotonic sodium chloride solution. After 0.5 hours the mice were killed and the tumor cells collected with a yield of 10.0 ml of packed cells. A perchloric acid extract of the tumor cells was prepared and after neutralization with potassium hydroxide, was chromatographed on a 1×20 cm column of Dowex-1-formate resin using a linear gradient elution system with 650 ml of 9.2 *M* formic acid in the reservoir.

sufficient to warrant further purification by chromatography. The chromatogram fractions containing the second peak of absorption at 323 mμ were pooled and evaporated to dryness under vacuum in a rotary evaporator with the bath temperature kept below 40° C. This procedure caused very little breakdown of the 6-MP metabolite.

The purified 6-MP metabolite was analyzed chemically for ribose and phosphate (8). The preparations contained no inorganic phosphate and, since only 20% of the phosphate was liberated by heating in 1 *N* H_2SO_4 at 100° C for 10 minutes, it was concluded that the phosphate present was not acid-labile. The base,[3] ribose, and phosphate components of this material were in the molar proportions of 1.00:1.14:1.20, respectively. Heating the metabolite in 1 *N* HCl for 1 hour at 100° C liberated free 6-MP which was identified by paper chromatography. This procedure destroyed 15% of the 6-MP.

The analytical data indicated that the second peak of 323 mμ absorption was due to a metabolite of 6-MP, probably 6-MP riboside monophosphate. This compound has been tentatively identified as a nucleoside-5′-monophosphate on the basis of its susceptibility to hydrolysis by snake venom

[3]The assumption was made (and subsequently shown to be correct) that the molar absorptivity of 6-MP in the nucleotide form was the same as that for the free base; namely 21.3×10^3 (10).

enzymes. When incubated with crude rattlesnake venom,[4] the analogue nucleotide was hydrolyzed with the liberation of an amount of inorganic phosphate equivalent to the purine base (in a typical experiment, 0.55 μmoles of inorganic phosphate were released from 0.56 μmoles of 6-MP ribonucleotide). In this same experiment the venom enzymes did not release inorganic phosphate from a mixture of adenosine-2′- and 3′- monophosphate isomers. The molar equivalence of the base and the phosphate released by the venom enzyme confirmed the results of the chemical analyses above and formed the basis for the identification of this metabolite as 6-MP nucleoside-5′-monophosphate.

Inorganic phosphate was also released in the expected amount from 6-MP nucleotide upon incubation with prostatic phosphatase[5] at 37° C in 0.05 *M* acetate buffer, pH 5.4 (9). This enzyme appeared to hydrolyze 6-MP nucleotide less readily than the mixture of adenosine-2′- and 3′-monophosphates, since a 1:100 dilution of the particular enzyme preparation employed rapidly liberated phosphate from the adenosine monophosphates, but did not hydrolyze the 6-MP compound appreciably. Under similar conditions, but with a 25-fold increase in the enzyme concentration, 6-MP ribotide was completely hydrolyzed liberating an amount of phosphate exactly equivalent to the base present.

In a similar experiment 6-MP nucleotide was incubated with prostatic phosphatase and, after being deproteinized with perchloric acid, the reaction mixture was chromatographed on Dowex-1-formate ion-exchange resin. Material, presumed to be 6-MP riboside, was located as a peak of 323 mμ absorption close to the expected position of 6-MP. This substance, after recovery from the eluate, contained 6-MP and ribose in equivalent molar proportions (base, 1.00; ribose, 0.99).

The absorption spectrum of 6-MP nucleotide is shown in Fig. 2. The values of the molar extinction coefficients were calculated from the ribose content of the solutions used for spectrophotometry. At pH values of 2 and 7, the absorption maxima of 6-MP nucleotide occurred at 323 mμ, whereas a maximum value at 327 mμ has been reported for the free base (10). The molar absorptivity of 21.3×10^3 for the nucleotide at pH 2 and pH 7 was found to be the same as the reported value for the free base (10). At pH 11 the peak of the absorption spectrum was at 311 mμ and the molar absorptivity was 20.1×10^3. The free base displays similar changes in the position of the absorption maximum and in the value of the extinction coefficient in alkaline solutions.

The synthesis of 6-MP nucleotide by Ehrlich ascites cells has also been demonstrated by measuring the incorporation of 6-mercaptopurine-8-C^{14} into this compound.[6] As may be seen in Fig. 3, 6-MP nucleotide was the principal metabolite of the labelled compound in the chromatogram fractions examined.

[4]Lyophilized venom from *Crotalus terrificus* was generously donated to Professor M. Darrach, Department of Biochemistry, by the Butanan Institute, Sao Paulo, Brazil. The hydrolysis was conducted at 37° C in a solution containing 1.7 mg of the crude, dried venom per ml, tris buffer (pH 8.6, 0.13 *M*), magnesium chloride (0.007 *M*), and the nucleotide substrate ($9–10\times10^{-6}$ *M*).

[5]The phosphatase enzyme was prepared by the method of Kerr and Chernigoy (9), from prostate glands obtained at autopsy, through the kind co-operation of Professor H. E. Taylor, Department of Pathology.

[6]6-Mercaptopurine-8-C^{14} was purchased from Isotope Specialties Company Inc., Burbank, Calif.

In this experiment the nucleotide derivative contained 13.6% of the injected radioactivity.

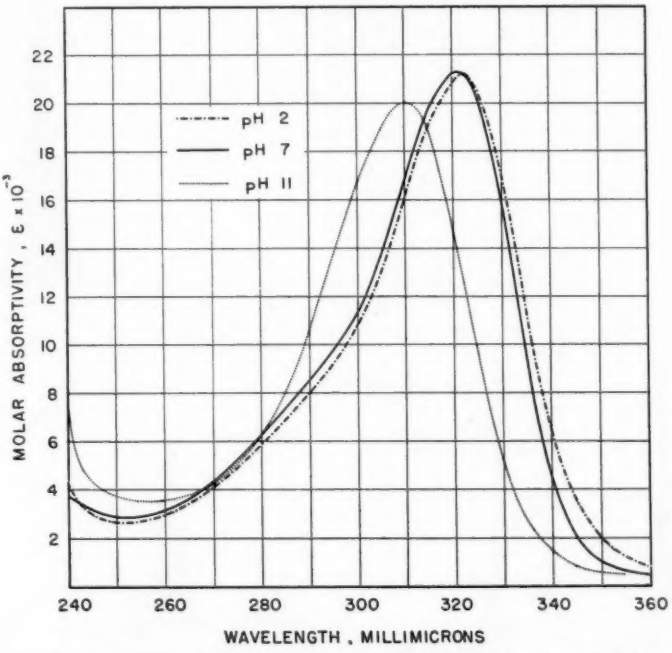

FIG. 2. The ultraviolet absorption spectrum of 6-mercaptopurine riboside-5′-monophosphate.

Conditions Affecting the in vivo Formation of 6-MP Riboside-5′-monophosphate

A study was made of conditions influencing the formation of 6-MP nucleotide by the ascites cells in response to a standard dose of 5 μmoles per 25 g mouse (approximately 30 mg per kg). As the test dose was injected directly into the ascitic fluid the initial relationship between the tumor cells and 6-MP approximated 4 μmoles per ml of packed cells. It may be seen in the data which follow that the dosage level of 6-MP does have some influence on the concentration of 6-MP ribotide formed, but two other factors have much greater effects: (*a*) the time interval between the test dose of 6-MP and the collection of the cells, and (*b*) the administration of 6-MP to tumor-bearing mice prior to the test dose of 6-MP (referred to as "pretreatment").

6-MP was very rapidly absorbed from the ascitic fluid and converted to 6-MP ribotide by the tumor cells. As may be seen in Table I, ascites cells collected 0.5 hours after the intraperitoneal administration of 6-MP had a higher concentration of the nucleotide metabolite than did cells collected at later intervals. Four hours after administration of 6-MP, the analogue and its nucleotide metabolite had disappeared completely from the tumor cells. These

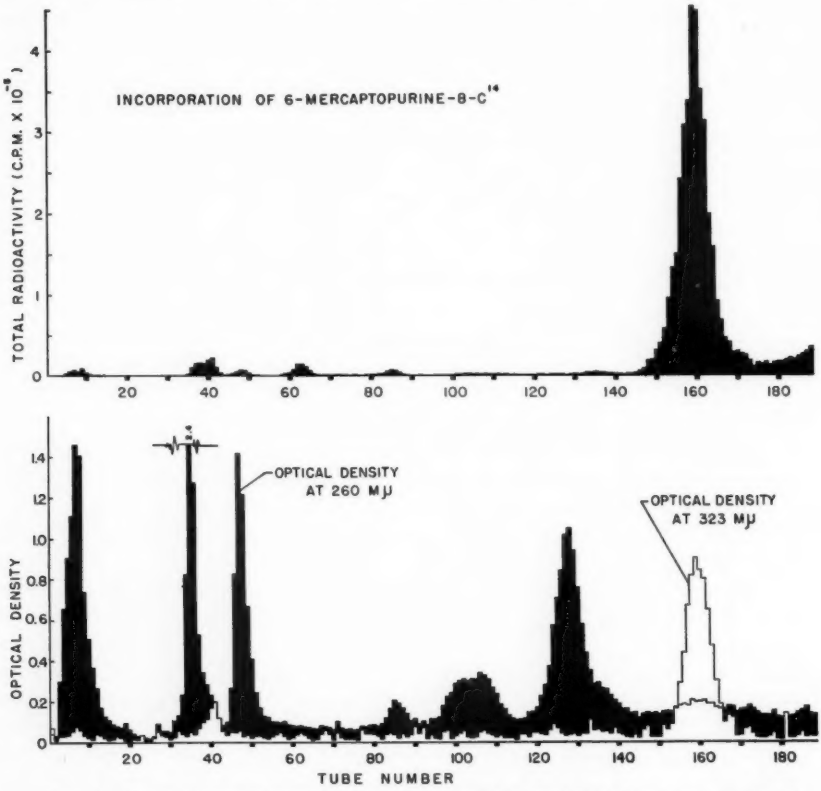

FIG. 3. Ion-exchange chromatogram of the soluble nucleotide fraction of Ehrlich ascites carcinoma cells treated in vivo with 6-mercaptopurine-8-C^{14}.

Seven days after tumor transplantation 10 mice were each injected intraperitoneally with 4 μmoles of 6-MP-8-C^{14} (specific activity, 1.05×10^6 c.p.m. per μmole) dissolved in isotonic sodium chloride solution. After 1.0 hour the mice were killed and the tumor cells collected (total yield, 6.7 ml packed cells). A neutralized perchloric acid extract of the tumor cells was chromatographed on a 1×20 cm column of Dowex-1-formate anion exchanger using a linear gradient elution system with 650 ml of 9.2 *M* formic acid in the reservoir. The ultraviolet absorption of each fraction was measured at 260 and 323 mμ and aliquots of each fraction were assayed for radioactivity.

The mice used in this experiment were treated with 6-MP on days 3, 4, 5, and 6 of tumor growth as described in Table II. The test dose of 6-MP-8-C^{14} was administered 24 hours after the last "pretreatment".

experiments indicated that 6-MP nucleotide was formed rapidly in the tumor cells, the bulk of the synthetic reaction occurring within 0.5 hours. Thereafter, the nucleotide derivative was catabolized by the tumor cells at a steady rate.

Effect of Pretreatment with 6-MP

It was observed that tumor cells which had been exposed to 6-MP prior to the test dose of 6-MP had a greatly increased capacity to form 6-MP nucleotide. Under the conditions described in Table II, this "pretreatment" effected a fivefold to sixfold increase in the formation of 6-MP nucleotide in the ascites

TABLE I

The formation of 6-MP nucleotide in untreated Ehrlich ascites carcinoma cells in vivo

Time interval between test dose and collection of cells (hours)	Test dose of 6-MP (μmole per ml packed cells)	6-MP nucleotide formed (μmole per ml packed cells)
0.5	4.8	0.13
	5.1	0.13
1.0	—*	0.091
	4.4	0.091
2.0	3.6	0.047
	4.0	0.059
	3.9	0.049†
4.0	4.9	0
	2.1	0
	5.0	0

*The test dose of 6-MP was 5 μmole per mouse, approximately the same dosage as in the other experiments.
†Data from A. Moyes and E. F. Ledgerwood.

TABLE II

The effect of pretreatment with 6-MP on 6-MP nucleotide formation in Ehrlich ascites carcinoma cells in vivo

Pretreatment* on indicated day of tumor growth	Test dose of 6-MP† (μmole per ml packed cells)	6-MP nucleotide formed‡ (μmole per ml packed cells)
None	3.9	0.049§
None	3.6	0.047
None	4.0	0.059
Day 3, 4, 5, 6	3.8	0.30§
Day 3, 4, 5, 6	3.3	0.30

*Pretreatment consisted of daily injections of a suspension of finely ground 6-MP: 5.25 mg of 6-MP per ml of isotonic sodium chloride solution, administered at the level of 42 mg per kg body weight.
†Test doses of 6-MP dissolved in isotonic saline were administered 24 hours after the last pretreatment.
‡6-MP nucleotide formation was measured in cells collected 2 hours after administration of the test dose of 6-MP.
§Data from A. Moyes and E. F. Ledgerwood.

cells. Pretreatment of the tumor cells consisted of a series of intraperitoneal injections of finely ground 6-MP, suspended in isotonic saline, administered on days 3, 4, 5, and 6 of tumor growth. Since the analogue was administered as a suspension and not as a solution, it was necessary to determine how long after injection this material persisted in the peritoneal cavity. The data of Table III indicated that 6-MP had essentially disappeared from the ascitic fluid 12 hours after the last pretreatment injection. These data showed that 12 hours or more after the last pretreatment injection, measurements of 6-MP nucleotide formation in the tumor cells in response to a test dose of 6-MP did not include residual analogue nucleotide derived from the pretreatment schedule.

The time interval between the last pretreatment and the test dose of 6-MP had considerable influence on the ability of the tumor cells to form 6-MP ribotide as may be seen in Table IV. As 6-MP ribotide formation appeared to be maximal about 24 hours after the last pretreatment and, as indicated above, no residual 6-MP nucleotide from the pretreatment injections was present this

TABLE III

The persistence of 6-MP in ascitic fluid

Interval between last treatment* and collection of cells (hours)	Concentration in micromoles per milliliter		
	Free 6-MP in ascitic plasma	Free 6-MP in cells	6-MP nucleotide in cells
8	0.02	0.01	0.18
12	Lost	0.03	Trace
24	0.0	0.005	0.0
24	0.01	0.016	0.0

*Each mouse received intraperitoneal injections of a suspension of 6-MP (dosage level as in Table II) administered on days 3, 4, 5, and 6 of tumor growth.

TABLE IV

The formation of 6-mercaptopurine nucleotide in Ehrlich ascites carcinoma cells in vivo; optimum time interval after pretreatment

Time interval between last pretreatment * and test dose of 6-MP (hours)	Test dose of 6-MP (μmole per ml of packed cells)	6-MP nucleotide† formed (μmole per ml of packed cells)
6	0.0 (Control)	0.18
	4.5	0.25
12	0.0 (Control)	0.0
	4.0	0.23
24	0.0 (Control)	0.0
	0.0 (Control)	0.0
	6.4	0.42
	3.3	0.35
	3.8	0.30‡
48	4.0	0.11
	4.1	0.18

*Pretreatment schedule as described in Table II.
†Animals were killed and the tumor cells collected 2 hours after the administration of the test dose of 6-MP.
‡Data from A. Moyes and E. F. Ledgerwood.

time, test doses of 6-MP were ordinarily administered 24 hours after the last pretreatment. As the number of pretreatment injections, whether 3, 4, or 5, did not appear to affect the rate of 6-MP nucleotide formation, the number of pretreatment injections routinely given was four.

In Table II, a comparison is made of the 6-MP nucleotide concentrations in pretreated and untreated tumor cells 2.0 hours after the injection of a test dose of 6-MP. This comparison exaggerates the effect of pretreatment since it was subsequently shown that 6-MP nucleotide was not only formed in larger amounts in pretreated tumor cells, but persisted at this elevated concentration for several hours, whereas that of the untreated cells decreased steadily after 0.5 hours. The experiments summarized in Table V indicated that within 0.5 hours after the test dose of 6-MP, the concentration of 6-MP nucleotide in pretreated cells rose to two to three times that of the untreated cells (see Table II) and continued at this level for at least two hours.

The present experiments do not attempt to account for the enhanced nucleotide formation in pretreated Ehrlich ascites carcinoma cells, but several

TABLE V

The formation of 6-MP nucleotide in "pretreated" Ehrlich ascites carcinoma cells in vivo*

Time interval between test dose of 6-MP and collection of cells (hours)	Test dose of 6-MP (μmole per ml of packed cells)	6-MP nucleotide formed (μmole per ml of packed cells)
0.5	4.2	0.34
1.0	3.6	0.25
	4.4	0.35
	6.6	0.30
2.0	3.3	0.35
	3.8	0.30
4.0	4.2	0.13

*Pretreatment schedule as described in Table II.

mechanisms may be suggested: (*a*) decreased catabolism of 6-MP nucleotide, (*b*) an induced (i.e. adaptive) formation of increased amounts of the pyrophosphorylase which is probably responsible for the formation of 6-MP nucleotide (1), and (*c*) an increased pool of phosphoribosylpyrophosphate (PRPP). The identity of the enzyme system responsible for the synthesis of 6-MP nucleotide in these experiments is unknown, but may be a pyrophosphorylase or a phosphorylase–kinase system. Lukens and Herrington have reported that the pyrophosphorylase activity of a beef liver preparation which converted guanine and hypoxanthine to their respective nucleotides also converted 6-MP to this form (1). An apparently separate pyrophosphorylase activity was responsible for the conversion of adenine to adenylic acid. The data of Table VI, which show that pretreatment of Ehrlich ascites cells with hypoxanthine did not significantly enhance the formation of 6-MP ribotide, suggested that pretreatment with 6-MP enhanced the formation of PRPP or perhaps a phosphorylase activity.

TABLE VI

The effect of pretreatment with hypoxanthine on the formation of 6-MP nucleotide

Pretreatment	Test dose of 6-MP (μmole per ml packed cells)	6-MP nucleotide formed (μmole per ml packed cells)
None	4.8	0.13
Hypoxanthine*	4.3	0.17

*Daily treatments with 5 μmole hypoxanthine per mouse on days 4, 5, and 6 of tumor growth.

Effect of Pretreatment with Azaserine

Sartorelli, LePage, and Moore (11) have reported recently that treatment of Ehrlich ascites carcinoma cells with azaserine enhanced the ability of these cells to convert thioguanine to thioguanosine monophosphate. Following this example, it was shown in the present experiments that azaserine pretreatment caused a threefold increase in the 6-MP nucleotide content of Ehrlich ascites cells. Six hours prior to the administration of a test dose of 6-MP, mice bearing this tumor received 2.0 mg of azaserine per kilogram of body weight by intra-

peritoneal injection. Thirty minutes after the test dose of 6-MP (3.7 μmoles per ml of packed cells), the tumor cells were collected and processed as described above. The 6-MP nucleotide content of these tumor cells was found to be 0.354 μmole per ml of packed cells, approximately three times that found in untreated cells under similar experimental conditions. Under the conditions described, pretreatment with 6-MP or azaserine produced increases of a similar magnitude in the 6-MP nucleotide content of Ehrlich ascites cells.

Azaserine is a potent inhibitor of the *de novo* pathway of purine nucleotide biosynthesis and is known to block this reaction sequence specifically at the conversion of formylglycinamide ribotide to formylglycinamidine ribotide (12). Moore and LePage (13) have shown that azaserine-treated ascites tumor cells accumulate formylglycinamide ribotide, indicating that the inhibited reaction in the tumor cells was similar to that in pigeon liver (12). Presumably, such a blockade would reduce the demand on the available pool of PRPP (and on the capacity of the tumor cell to synthesize PRPP) and thereby make increased amounts of this metabolite available to other reactions.

General Aspects of 6-MP Incorporation

In the present experiments the test doses of 6-MP were injected intraperitoneally and were, in effect, intratumoral injections. The tumor cells, exposed directly in this way to high concentrations of 6-MP, incorporated the analogue rapidly, as may be seen by the high concentrations of 6-MP nucleotide at the shortest time intervals (0.5 hour) listed in Tables I and V. An examination of free 6-MP and 6-MP nucleotide concentrations in the tumor cells and in the deproteinized ascitic plasma showed that 6-MP nucleotide was found only in the cellular components of the ascites fluid. At time intervals greater than 0.5 hours after the administration of 6-MP, the free base was found only in very small amounts within the tumor cells, both with and without pretreatment with 6-MP. It was apparent that 6-MP nucleotide was formed rapidly and that this compound was one of the principal metabolites of 6-MP in the tumor cells. Although the tumor cells apparently took up the analogue selectively from the surrounding plasma, they contained only a small part (2–14%) of the injected dose of 6-MP.

No significant peaks of absorption at 323 mμ other than those attributable to 6-MP and 6-MP nucleotide have been found on extended ion-exchange chromatograms of the acid-soluble fractions of 6-MP-treated Ehrlich ascites carcinoma cells. In the extended chromatograms the linear gradient elution system described in Fig. 1 was used as far as tube 170. Elution was continued from this point with a linear gradient system employing ammonium formate and form c acid.[7] With this extended system, adenosine triphosphate appeared between tubes 218 and 229. No significant peaks of absorption at 323 mμ appeared between that of 6-MP ribotide (tubes 139–158) and tube 354.

Similar chromatographic experiments showed that ascitic plasma from 6-MP-treated mice contained two substances with absorption at 323 mμ which were retained on Dowex-1 anion-exchange resin. One of these substances

[7]The mixing vessel contained 600 ml of 5.1 *M* formic acid and the reservoir contained a solution which was 5.1 *M* in formic acid and 1.5 *M* in ammonium formate.

was recognized as 6-MP by its position on the chromatograms. The other substance which was present in smaller amounts, followed the free base, appearing in tubes 45–49. No other peaks of 323 mμ absorption were apparent when the extended gradient system described above was continued as far as fraction 350.

6-MP Nucleotide Formation in Liver and Intestine

The synthesis of 6-MP nucleotide by normal tissues taken from tumor-bearing mice was investigated by the ion-exchange chromatographic methods described above and by a more sensitive method employing 6-MP-8-C^{14} combined with the "carrier" technique.

The 6-MP nucleotide peak was not apparent in the profiles of 323 mμ absorption for nucleotide chromatograms of liver and intestine taken from 6-MP-treated animals bearing the ascites tumor, whereas the latter tissue contained large amounts of 6-MP nucleotide. In a similar experiment, mice bearing subcutaneous implants of a solid tumor[8] were each injected intraperitoneally with 1 μmole of 6-MP-8-C^{14} and, after 1 hour, perchloric acid extracts of liver, intestine, and tumor were prepared. Prior to chromatography, non-isotopic 6-MP nucleotide ("carrier") was added to each extract. After recovery from ion-exchange chromatograms of these extracts, the carrier nucleotide was found to be radioactive in each case, indicating that 6-MP nucleotide was derived from 6-MP-8-C^{14} in each tissue. The amount of nucleotide synthesized was small in each case and would not have been detectable by light absorption measurements at 323 mμ.

Samples of the recovered nucleotides were hydrolyzed by heating in 1 *N* HCl for 1 hour at 100° C and the liberated 6-MP was recovered by paper chromatography. In each case the free base was radioactive, indicating that the radioactivity observed in the carrier nucleotide was present in the form of 6-MP and was not due to accompanying contaminants. As may be seen in Table VII the amounts of 6-MP nucleotide detected in these tissues were small, being 2–6% of that found in the ascites cells.

6-MP Nucleotide Synthesis in vitro

The formation of 6-MP nucleotide in vitro was demonstrated in experiments in which the ascites tumor cells were incubated with 6-MP-8-C^{14} under conditions which permitted anaerobic glycolysis. The composition of the medium had considerable influence on the synthesis of 6-MP nucleotide. As may be seen in Table VIII, cells which were incubated in a medium consisting of 2 parts of ascitic plasma and 1 part of Robinson's medium,[9] contained 1.5 times the amount of 6-MP nucleotide found in cells incubated in Robinson's medium alone. The level of 6-MP nucleotide synthesis in the glycolyzing tumor cells approached that found under in vivo conditions. Approximately 21% of the

[8]This tumor, a 6-MP-resistant variant of Sarcoma 180, and the ascites form of Sarcoma 180 were obtained through the courtesy of Dr. D. A. Clarke of the Sloan-Kettering Institute for Cancer Research.

[9]Robinson's medium (14) was modified for anaerobic glycolysis by reducing the NaCl concentration to 0.109 *M* and by adding $NaHCO_3$ (0.025 *M*) and glucose (0.0056 *M*). The incubation was conducted in flasks with a gas phase of 95% nitrogen and 5% carbon dioxide. (LePage, G. A. Personal communication.)

TABLE VII

The formation of 6-MP nucleotide in liver, intestine, and tumor*

	μmole of 6-MP nucleotide formed per g fresh tissue†
Liver	6.1×10^{-3}
Intestine	2.9×10^{-3}
Tumor	2.1×10^{-3}

*A 6-MP-resistant subline of S-180. See footnote[8].

†Tissues were collected from four mice 1 hour after each had received an intraperitoneal injection of 1 μmole of 6-MP-8-C^{14}. Each mouse had bilateral, subcutaneous implants of tumor. The acid-soluble extracts of the three tissues were prepared as described above and 0.6 μmole of non-isotopic 6-MP nucleotide was added to each extract as carrier prior to chromatography. 6-MP nucleotide synthesis in each tissue was calculated on the basis of the radioactivity present in the recovered carrier nucleotide.

TABLE VIII

The formation of 6-MP nucleotide in Ehrlich ascites carcinoma cells in vitro*

	6-MP nucleotide formed
Cells incubated in Robinson's medium	0.38 μmole per ml packed cells
Cells incubated in ascites plasma plus Robinson's medium	0.57 μmole per ml packed cells

*Ehrlich ascites carcinoma cells, which had been treated with 6-MP during growth ("pretreatment", see Table II), were washed once with cold Robinson's medium (14) and resuspended in this medium alone or in ascites plasma (2 parts) plus Robinson's medium (1 part) in the proportion of 2.0 ml of packed cells per 12.0 ml of suspension. The ascitic plasma was fortified by the addition of glucose and sodium bicarbonate (100 and 200 mg per 100 ml, respectively). The incubation was conducted in 125-ml flasks each containing 12.0 ml of the cell suspension and 1.35 μmole of 6-MP-8-C^{14} (4.43×10^{6} c.p.m.). The flasks were gassed with 95% nitrogen – 5% carbon dioxide and were incubated at 37° C for 30 minutes. The contents of duplicate flasks for each medium were pooled and perchloric acid extracts prepared for chromatography as described above.

labelled 6-MP substrate was converted to the nucleotide form in the tumor cells which were incubated in the plasma-containing medium.

6-MP Nucleotide Synthesis by the Ascites Form of Sarcoma 180

The growth of the Ehrlich ascites carcinoma may be greatly reduced by treatment with 6-MP. In view of the coincidence of sensitivity to 6-MP and the ability of this tumor to form 6-MP nucleotide, it was considered desirable to investigate 6-MP nucleotide formation in Sarcoma 180, a tumor which is particularly sensitive to 6-MP therapy (15). The formation of 6-MP nucleotide was demonstrated in the ascites form of Sarcoma 180,[8] using the chromatographic techniques described above. The nucleotide metabolite formed by this tumor was presumed to be the same as that found in the Ehrlich carcinoma cells on the basis of its position on the ion-exchange chromatogram. Sarcoma 180 ascites cells collected 40 minutes after the injection of 6-MP (3.5 μmoles per ml of packed cells) contained 0.14 μmoles of 6-MP nucleotide per ml of packed cells. This tumor and the Ehrlich ascites carcinoma appear to have similar capacities for the synthesis of the analogue nucleotide.

Nucleotide formation appears to be a general metabolic reaction with the biologically active compounds of this class. The possibility that 6-MP and other purine analogues may act at the nucleotide level of metabolism, perhaps as false coenzymes, has been suggested by Way and Parks (3). This concept is supported by the present demonstration in two 6-MP-sensitive mouse neoplasms[10] of the ability to convert this compound to 6-MP nucleotide.

[10]A 6-MP-resistant subline of the Ehrlich ascites carcinoma which is unable to convert 6-MP to the nucleotide form has been developed in this laboratory. (Paterson, A. R. P. and Ledgerwood, E. F. Unpublished results.)

Acknowledgment

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ZONE ELECTROPHORESIS OF TOBACCO MOSAIC VIRUS[1]

P. M. TOWNSLEY

Abstract

An apparatus is described for the preparative zone electrophoresis of tobacco mosaic virus in agar gel. The isolated virus does not have any detectable ribonuclease activity.

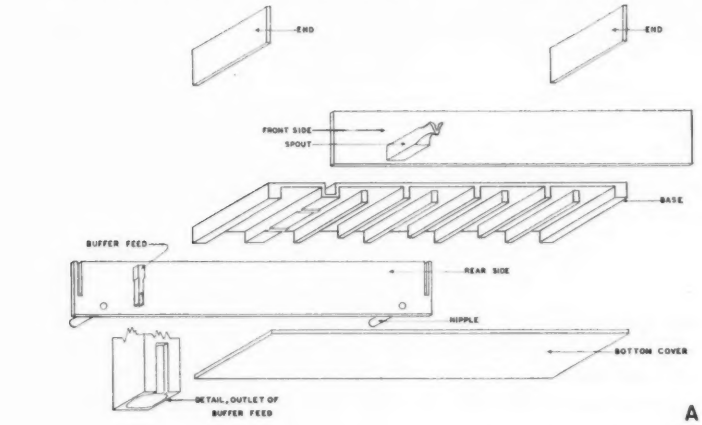
Introduction

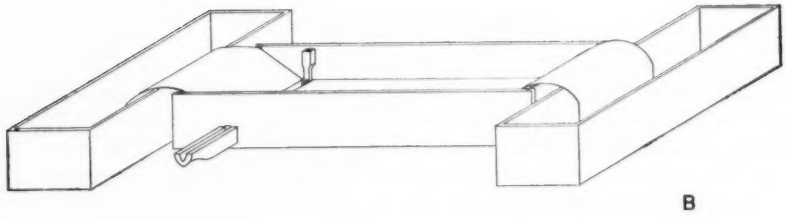
A previous paper (1) has described the separation of tobacco mosiac virus (TMV) from crude preparations by electrophoresis in agar gel. It was noted that the virus migrated as a rather well-resolved narrow band. This suggested that, if an apparatus were devised in which the migrating bands could be eluted from the gel, a preparation of virus might be obtained relatively free of contamination. This paper describes an apparatus which elutes the constituents when they reach a predetermined point on the agar sheet.

Apparatus and Materials

The apparatus was made from Perspex sheets and consisted of three units: an electrophoresis bed and two electrode vessels. The base of the electrophoresis bed was fabricated from a sheet 30×8×1.3 cm from which was cut a water-cooling chamber with baffles and a sample-collecting trough (Fig. 1A). The sides, 30×4.5×0.3 cm, were cemented to the long edges of the base providing the sides to the cooling chamber and to the electrophoresis bed. Similarly a sheet 30×8.6×0.3 cm was cemented to the bottom of the base to seal the cooling chamber. Removable end pieces were cut from 0.3-cm sheet to fit into the retaining slots located at the ends of the side pieces. Two holes, 0.8-cm diameter, were cut through both ends of the rear side into the cooling chamber and Perspex nipples inserted to provide cooling water connections. A rectangular hole 1×0.3 cm was cut through the front side into the sample-collecting trough to provide continuous removal of the sample. In addition in order to conduct the sample away from the side of the apparatus and also to maintain a constant liquid level within the collecting trough, a spout was fashioned from Perspex and cemented to the rectangular hole. A rectangular piece of Perspex 0.6×0.6×3 cm containing a hole 0.3 cm in diameter bored down its length was cemented to the inside of the rear side. This tube served as a receptor for a glass capillary constant-flow regulator and as an entrance for buffer into the sample-collecting trough. The electrophoresis bed was supported on a platform that could be moved up or down between the electrode vessels to counteract capillary flow of the buffer to or from the electrophoresis medium.

[1]Manuscript received January 16, 1959.
Contribution No. 10, Research Station, Canada Agriculture, c/o The University of British Columbia, Vancouver 8, British Columbia.





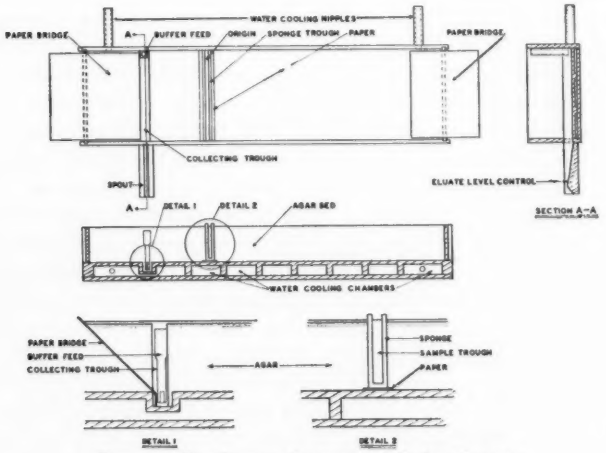


FIG. 1. Continuous electrophoretic fractionator.

The two electrode vessels, 20×7×5 cm, were constructed from 0.3-cm sheet (Fig. 1B). The electrodes were of platinum wire enclosed in 1/4-in. dialysis tubing through which buffer solution flowed slowly to remove the electrode reaction products.

In preparing the electrophoresis bed for the agar medium, a strip of 3 mm Whatman filter paper was cut and folded to fit the spout as a lining reaching just inside the wall of the tray. A temporary dam of filter paper prevented the fluid agar from escaping from the tray into the spout. The electrophoresis bed was bridged to the electrode vessels with two thicknesses of 3 mm filter paper strips as shown in Fig. 1C.

The electrophoresis bed was filled with a hot agar solution of 0.7% agar[2] containing 0.02 *M* tris(hydroxymethyl)aminomethane and 0.02 *M* succinate (tris–succinate), previously adjusted to pH 7.0 with sodium hydroxide.[3] While the agar was still liquid, a narrow rectangular trough with an inside dimension of 1.5 mm was fashioned from a strip of sponge plastic,[4] 8×0.6×2 cm. A trough of 3-mm inside dimensions was used for larger samples. This was soaked in the hot agar solution to remove trapped air bubbles and laid in the tray parallel to the groove and about 4 cm from it. A strip of filter paper slightly wider than the sponge was placed under the sponge on the bottom of the tray. This was done before the sponge was set in place in the hot agar. A strip of Perspex was cut to fit the trough in the sponge and inserted into it until the agar had hardened. When the gel was set this strip was removed and the trough two-thirds filled with the plant sap or solution to be tested. At the same time, a narrow channel parallel to the sponge trough was cut in the agar above the groove leading to the spout and the agar in the groove removed. The temporary dam of filter paper was removed but not the filter paper lining of the spout which was now impregnated with agar that had seeped past the dam; the excess of agar in it was removed so that the channel was clear from the agar bed to the end of the spout. The bottom of the channel in the spout was slightly higher than the groove itself. Buffer solution was kept slowly passing down the channel, at a constant flow of 15 ml per hour, from the end farthest from the channel spout. The buffer filled the channel and overflowed from the end of the spout where 5-ml aliquots were collected with a model VIO Gilson Medical Electronic fraction collector.

Experimental

Two experiments were performed to test the apparatus. The optical density of the eluate was determined either continuously using the Gilson Medical Electronic model 10 absorption meter, or with a Beckman DU Spectrophotometer. The radioactive disintegration rate was measured with a Geiger–Müller tube TGC 1/1B83 and a model 2105 Berkeley scaler on 4-ml aliquots dried to an "infinite thickness" on planchets. The infectivity of the eluate was tested by

[2]Higher agar concentrations decrease the electrophoretic mobility of TMV.

[3]0.025 *M* tris(hydroxymethyl)aminomethane – hydrochloric acid buffer at pH 7.5 may also be used.

[4]Unifoam, a trade name for a Urethane foam manufactured by W. T. Burnett Co. Inc., 1500 Bush St., Baltimore 30, Maryland, U. S. A.

quantitative half-leaf local lesion assay on *Nicotiana glutinosa* L., and by inoculation of tobacco. Flow birefringence was measured with an apparatus manufactured by Rao Instrument Co., Brooklyn 5, N. Y. The technique for the determination of ribonuclease activity and labile phosphate was that described by Holden and Pirie (2) and by Pirie (3) with the exception that uranyl acetate – trichloroacetic acid reagent was used as a precipitant for the detection of labile phosphate. Phosphorus was determined by the procedure of Michelsen (4). Protein was determined by the biuret reaction (5).

Experiment 1

The top leaves of tobacco plants, *N. tabacum* L. var. Haronova, were harvested intact after approximately two weeks' infection with a common strain of TMV (1). The excised leaves (about 120 g) were allowed to metabolize radioactive phosphate by immersing the petioles in nutrient solution (1) containing 1 μc of P^{32} orthophosphate per ml for 4 hours. Seventy milliliters of juice were expressed from the labelled leaves with mortar and pestle at 0° C and centrifuged at 6000×*g* in a Servall centrifuge for 10 minutes to remove the debris. The supernatant was centrifuged at 75,000×*g* in a Spinco model L ultracentrifuge for 90 minutes, and the pellet containing TMV was taken up in 1 ml of 0.02 *M* tris – succinate pH 7.0. This was transferred to the origin position of the electrophoresis apparatus, located 4 cm from the collecting trough. The sample was fractionated in an electric field strength of 5 volts per cm (150 volts potential at the electrodes with a current of 15 ma). The eluate, collected in 5-ml aliquots, was tested for optical density at 260 mμ, radioactivity, flow birefringence of every third aliquot, and infectivity of every eighth aliquot.

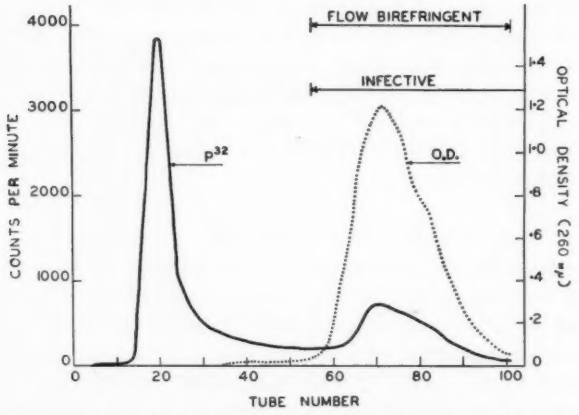


FIG. 2. The electrophoretic isolation of P^{32}-labelled tobacco mosaic virus.
The P^{32}-labelled TMV was purified by one alternate slow and high speed centrifugation (1), then separated by agar electrophoresis in 0.7% agar containing 0.02 *M* tris–succinate buffer at pH 7.0. The electric field strength was 5 volts per cm (150 volts potential at the electrodes with a current of 15 ma). The eluate was collected in 5-ml aliquots at 15 ml per hour.

Results

The results of fractionating P^{32}-labelled TMV are shown in Fig. 2. The plot of optical density of the eluate versus tube number showed a single skewed absorption peak, but the plot of radioactivity in counts per minute versus tube number showed two peaks. These were: a sharp peak in tubes 16 to 23 and a broad peak in tubes 60 to 100. The sharper peak was due to contamination with radioactive inorganic phosphate and the broader peak was due to incorporation of P^{32} into tobacco mosaic virus. The increase in optical density was accompanied by a proportional increase in radioactivity. Flow birefringence, demonstrating the presence of long particles was found only in tubes 56 to 104. Material causing typical TMV symptoms when inoculated into indicator plants occurred in tubes 56 to 143, which was the last tube collected.

Experiment 2

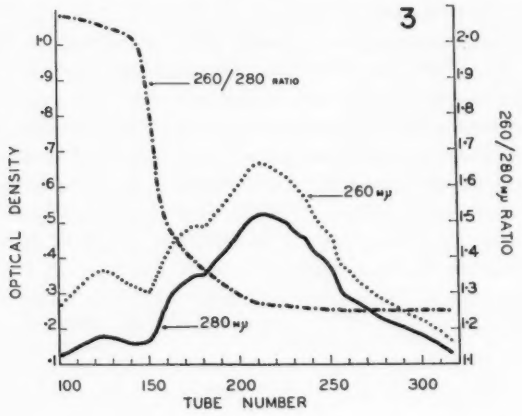
Pirie (6) has stated that in all so-called purified TMV preparations he has examined, the enzyme ribonuclease remained associated with the virus. To test the purification accomplished by the present technique, the second experiment was set up to determine the ribonuclease activity, and infectivity per mg of protein.

A TMV preparation containing 210 mg of protein, which had been purified by means of two cycles of alternate high and slow speed centrifugation at 75,000$\times g$ for 90 minutes and 6000$\times g$ for 10 minutes respectively, was taken up in 4.0 ml of 0.025 *M* tris – HCl pH 7.5 buffer and a 2.5-ml aliquot was placed into the zone electrophoresis apparatus. The remaining 1.5 ml was stored at 0° C. The electric field strength was 5 volts per cm (150 volts potential at the electrodes with a current of 18.5 ma).

A pattern was obtained similar to Fig. 2 when the eluate was measured at an optical density of 260 mμ. The optical density of every fifth tube beginning at tube 100 at 260 mμ and 280 mμ is recorded in Fig. 3. The plot of the change in the 260/280 ratio is given to show the separation of two materials. The absorption spectra of typical tube contents in buffer solution from the material of high 260/280 ratio and from material of low constant 260/280 ratio are given in Fig. 4.

The eluates in tubes 160 to 208 and 209 to 260 were combined and ultracentrifuged at 75,000$\times g$ for 90 minutes. The resultant pellets, labelled A and B respectively, were taken up in water and centrifuged at 6000$\times g$ for 10 minutes. At the same time the original 1.5-ml sample that had been stored at 0° C and had not been submitted to electrophoresis was ultracentrifuged again. The pellet was taken up in water and centrifuged at slow speed. A tabulation of the amount of labile phosphate, and the ribonuclease activity as measured by the phosphorus soluble after uranyl acetate – trichloroacetic acid precipitation is shown in Table I.

The yield of TMV from agar gel electrophoresis, based on protein analyses, ranged from 50 to 60% when compared with thrice ultracentrifuged control samples. Complete recovery of the protein added to the gel could not be



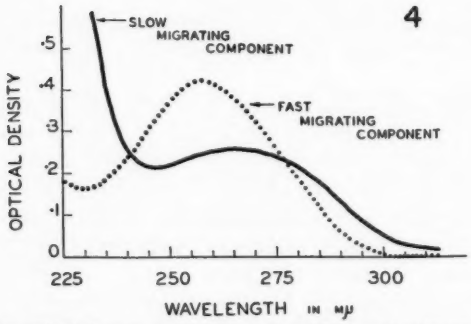


FIG. 3. The optical density at 260 mμ and at 280 mμ of tobacco mosaic virus isolated by agar gel electrophoresis.

The TMV was purified by two alternate slow and high speed centrifugations (1), then separated by electrophoresis in 0.7% agar containing 0.025 *M* tris – HCl buffer at pH 7.5. The electric field strength was 5 volts per cm (150 volts potential at the electrodes with a current of 18.5 ma). The origin was 4.3 cm from the collecting trough. The eluate was collected in 5-ml aliquots at 19 ml per hour.

FIG. 4. Optical density of two eluted fractions isolated from a so-called purified TMV preparation by continuous agar gel electrophoresis.

obtained since a large proportion of the protein either did not migrate in the direction of the elution trough or migrated at a much slower rate than TMV. The material which remained in the origin position after electrophoresis was still highly infectious. The infectivity of fractions A and B did not differ significantly from the stored ultracentrifuged TMV.

The amount of labile phosphate is a measure of the ability of the preparation to autolyse if incubated for 24 hours in citrate buffer at 37° C. No labile phosphate was observed showing that the preparation was stable.

Ribonuclease activity was located only in the TMV preparation that had not been isolated by zone electrophoresis.

TABLE I

Labile phosphorus and ribonuclease activity in ultracentrifuged TMV and in ultracentrifuged TMV after agar gel electrophoresis

Sample	Labile phosphate, μg/mg protein		Phosphorus soluble in UrTCA, μg/mg protein	
	0 hr	24 hr	0 hr	24 hr
Twice-ultracentrifuged TMV stored at 0° C and reultracentrifuged	0.0	0.0	0.0	2.8
Combined ultracentrifuged pellets from eluate tubes Nos. 160 to 208	0.0	0.0	0.0	0.0
Combined ultracentrifuged pellets from eluate tubes Nos. 209 to 260	0.0	0.0	0.0	0.0

NOTE: Reaction mixture = 2 mg TMV protein + 2 mg yeast sodium ribonucleate (pH 6.0) + 0.2 ml of 0.1 *M* citrate (pH 6.0) + a few crystals of thymol + H_2O to 2.0 ml final volume. Samples were withdrawn at 0 and 24 hours and precipitated with uranyl acetate and TCA reagent (3). Phosphorus was determined on the supernatant. Labile phosphate was determined in the same manner except yeast nucleic acid was not added.

The radioactivity of the isolated TMV which had been previously exposed for short periods to P^{32} orthophosphate is now under study in order to determine whether there is any difference in the rate of incorporation into these fractions.

Discussion

The agar gel electrophoresis apparatus described has proved successful for the isolation of TMV from so-called purified preparations. The absence of ribonuclease activity in ultracentrifuged TMV preparations that have been submitted to zone electrophoresis indicates that the enzymatic activity may not be combined with TMV but merely associated with it in ultracentrifuged pellets. The presence of ribonuclease in TMV preparations has been given as a possible reason for the rapid decrease in infectivity of TMV nucleic acid (6).

Acknowledgments

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THE COMBINATION OF COPPER WITH AMINO ACIDS, PEPTIDES, AND PROTEINS[1]

J. F. SCAIFE[2]

Abstract

By equilibrating solutions containing amino acids, peptides, or proteins with the sparingly soluble copper salt malachite ($CuCO_3.Cu(OH)_2$), it has been possible to determine the amount of copper in the solutions complexed to these compounds, and the amount of free copper in the solutions in equilibrium with the complex. The dissociation constant for the copper–glycine complex has been estimated from the data obtained using this system. The nature of the complexes has been deduced both from chemical determinations of the bound copper, and from manometric measurements of the extent of complexing, made in bicarbonate buffers. The simple amino acids have been shown to form complexes of the type CuR_2, which are appreciably dissociated in dilute solutions. The degree of complexing is influenced by the nature of the group R. Histidine, tryptophane, and other compounds containing more than one donor nitrogen atom are able to form more than one type of complex with copper. The participation of other nitrogen atoms in complexing is related to their basicities. Glycylglycine is able to bind approximately twice as much copper as glycine, but the other glycine peptides from triglycine to pentaglycine show a reduced and progressively decreasing ability to bind copper.

The binding of copper to proteins differs from that of the amino acids, in that the amount of copper bound is independent of the concentration of protein present, for any given concentration of free copper. The several atoms of copper bound to each protein molecule were not all bound with the same affinity.

Introduction

It is a matter of interest to learn how a metal, whether occurring naturally or acquired as a contaminant, distributes itself between such constituents of a biological system as amino acids and proteins, what are the catalytic powers of these compounds, and how closely their properties approach those of the specific metal-containing enzymes. Towards this end, a study has been made of the mode of combination of copper with certain amino acids, peptides, and proteins. In a subsequent paper the results of a study of the activity of the compounds in catalyzing the aerobic oxidation of L-ascorbic acid are reported.

Biological systems often contain bicarbonate and phosphate ions which can form sparingly soluble salts with copper. Numerous other compounds, even buffer constituents like acetate or citrate, are able to form complexes in solution with copper ions. In an attempt to avoid the difficulty of determining the distribution of copper between the other constituents of the system when a cupric salt is added to a solution of an amino acid or protein in a buffer, the device has been adopted of equilibrating the solution of amino acid or protein with a sparingly soluble salt of copper. Malachite ($CuCO_3.Cu(OH)_2$) was chosen for this purpose because it is apparently the least soluble and hence

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presumably the most stable (in solution) of the naturally occurring carbonates of copper.

By this means, it was possible to define the concentration of cupric ions in the solution. When the solution contained an amino acid or a protein, an excess of copper found in the solution above the amount calculated from the solubility product of malachite (1) (or determined experimentally in the absence of the complexing agent) then gave the amount of complexed copper. This system proved, however, to be rather less simple than was anticipated, since evidence was obtained that cupric ions are able to form a complex with bicarbonate ions (1).

An extensive literature exists dealing principally with the combination of copper with amino acids, proteins, and related compounds (2–25). A discussion of the principles involved in the various methods utilized for making these investigations has been published (26, 27); of special relevance to the present work may be mentioned those methods utilizing a sparingly soluble copper salt as a source of copper (11, 13).

Methods and Materials

The combination of copper with amino acids and related compounds has been investigated mainly by chemical means (28, 29), but also manometrically in the Warburg apparatus. The chemical investigations are more sensitive at low concentrations of amino acid (of the order of 5×10^{-5} *M*) and it was found that the manometric changes observed when malachite reacted with an amino acid in a bicarbonate – carbon dioxide buffer were difficult to interpret, by themselves, in terms of bound copper.

The determination of the solubility product and the preparation of malachite has been previously described (1). The techniques and apparatus used in measuring the complexing of amino acids, peptides, and proteins with the copper derived from malachite were the same as those described for the determination of the solubility product. All measurements were made at 25° C.

The gas mixtures used consisted of carbon dioxide in nitrogen or else pure carbon dioxide.

The amino acids were tested for purity by two-dimensional paper chromatography and where necessary were purified by recrystallization.

The di- and tri-peptides were commercial products (Roche), while the tetra- and penta-peptides were synthesized by a standard procedure (30).

The proteins were crystallized products supplied by Armour and Co. Ltd. In calculating molarities, allowance was made for their moisture content.

The compounds α,∂-diaminoadipic acid, and 5-amino-*n*-caproic acid were synthesized according to published procedures (31, 32, 33, 34).

The Equilibration of Malachite with Solutions of Amino Acids

(*a*) *Chemical Determination*

As was pointed out previously (1), there is a need to use not less than 50 mg of malachite per 50 ml of solution for equilibration studies. If less is used, especially with solutions containing amino acids, there is a tendency for the

eroded particles of malachite to become colloidal. When such solutions were incubated for several days the suspended particles increased in size and finally precipitated, so that reproducible results were obtained. On the other hand, an increase in the relative amount of malachite much above the level of 50 mg per 50 ml of solution resulted in the adsorption, in these dilute solutions, of significant amounts of copper – amino acid complex by the solid.

The method finally adopted is illustrated by a specific example: 50 mg of malachite was weighed into the equilibration vessel; 27.5 ml of water, 10 ml of 0.2 *M* sodium bicarbonate, and 10 ml of *M* sodium chloride were added and the vessel was shaken in the water bath at 25° C for 15–20 minutes while a stream of the gas mixture was passed through the vessel. The solution of the amino acid (2.5 ml adjusted to the final pH of the buffer) was added slowly. Shaking and the passage of gas (at 6 liters per hour) was continued for 1 hour. The taps of the vessel were then closed and equilibration allowed to proceed for 12–14 hours. At the end of this period, the gas mixture was again passed through the vessel for 1–2 hours, after which a filtered sample was removed for chemical analysis.

(*b*) *Manometric Technique*

As previously mentioned, the manometric investigations were restricted to relatively concentrated solutions of amino acids (of the order of 0.025 *M*). In most instances parallel experiments were made under the same conditions making use of the apparatus used for the chemical determinations. The filtered solution was then used for copper determinations and the measurement of retention. Methods for the estimation of the retention by a given solution over the range of pH change to be expected in the actual reaction have been described (35–37). In the present work the "zweites prinzip" of Warburg (38) has been used. Total retention was found in all cases to be negligible when the pH of the solution was less than approximately 7.6.

In the manometric measurements, the ionic strength of the bicarbonate buffer was maintained constant at 0.2 by the addition of the appropriate amount of sodium chloride.

Results

The Analytical Determination of the Combination of Copper with Amino Acids, Peptides, Proteins, and Related Compounds

Unless otherwise stated, the final concentration of sodium bicarbonate for equilibration in the bicarbonate – carbon dioxide buffer was 0.04 *M*, and that of sodium chloride was 0.2 *M*.

Amino Acids and Peptides

The results of the analyses are given in Table I. In some instances the compound was equilibrated with a sample of malachite which possessed a slightly higher solubility than normal. In these instances the sub-tables of Table I are marked with an asterisk, and the results are strictly comparable only among themselves. Variations in the solubility of sparingly soluble salts are well known (39).

TABLE I

Combinations of copper with amino acids and peptides

C_a	Cu_b	Cu_b/C_a	C_a	Cu_b	Cu_b/C_a	C_a	Cu_b	Cu_b/C_a	C_a	Cu_b	Cu_b/C_a
Glycine Cu_f 0.0323 (2.05 μg per ml) *P* 0.969; pH 6.309			Glycine Cu_f* 0.0356 (2.26 μg per ml) *P* 0.775; pH 6.406			Glycine Cu_f 0.0185 (1.17 μg per ml) *P* 0.410; pH 6.682			Glycine Cu_f* 0.0230 (1.46 μg per ml) *P* 0.368; pH 6.730		
0.1	0.028	0.280	0.1	0.0305	0.305	0.1	0.0171	0.171	0.1	0.0265	0.265
0.2	0.0508	0.254	0.2	0.0604	0.302	0.2	0.0432	0.216	0.2	0.0578	0.289
0.3	0.0858	0.286	0.5	0.158	0.315	0.3	0.0735	0.245	0.3	0.0894	0.298
0.5	0.155	0.310				0.5	0.131	0.262	0.5	0.161	0.322
5.0	1.980	0.396							1.0	0.362	0.362
									10.0	3.69	0.369
Glycine Cu_f 0.0147 (0.937 μg per ml) *P* 0.194; pH 7.008			Glycine Cu_f* 0.0116 (0.737 μg per ml) *P* 0.0485; pH 7.610			DL-α-Alanine Cu_f* 0.0235 (1.50 μg per ml) *P* 0.388; pH 6.706			β-Alanine Cu_f* 0.0235 (1.50 μg per ml) *P* 0.388; pH 6.706		
0.1	0.0184	0.184	0.1	0.0244	0.244	0.1	0.0221	0.221	0.5	0.00925	0.0185
0.2	0.0428	0.214	0.2	0.0584	0.292	0.2	0.0428	0.214	1.0	0.0158	0.0158
0.3	0.0759	0.253	0.3	0.0933	0.311	0.3	0.0663	0.221	2.0	0.0264	0.0132
0.5	0.160	0.319	0.5	0.171	0.341	0.5	0.118	0.235	3.0	0.0396	0.0132
DL-Glutamic acid Cu_f* 0.0235 (1.50 μg per ml) *P* 0.388; pH 6.706			L-Glutamic acid Cu_f* 0.0235 (1.50 μg per ml) *P* 0.388; pH 6.706			L-Glutamic acid Cu_f 0.0179 (1.14 μg per ml) *P* 0.388; pH 6.706			DL-Tryptophane Cu_f* 0.0235 (1.50 μg per ml) *P* 0.388; pH 6.706		
0.2	0.0476	0.238				0.1	0.0272	0.272	0.1	0.0370	0.370
0.3	0.0834	0.278	0.3	0.0972	0.324	0.2	0.0516	0.258	0.2	0.0775	0.385
0.5	0.139	0.277	0.5	0.154	0.308	0.3	0.0894	0.298	0.3	0.119	0.395
1.0	0.318	0.318				0.5	0.125	0.250	0.5	0.145	0.289
DL-Proline Cu_f* 0.0235 (1.50 μg per ml) *P* 0.388; pH 6.706			DL-β-Phenylalanine Cu_f 0.0180 (1.15 μg per ml) *P* 0.390; pH 6.704			DL-Lysine Cu_f 0.0322 (2.05 μg per ml) *P* 0.969; pH 6.309			DL-Lysine Cu_f 0.0250 (1.59 μg per ml) *P* 0.775; pH 6.406		
0.1	0.0126	0.126	0.1	0.0375	0.375	0.1	0.0260	0.260	0.1	0.0218	0.218
0.2	0.0324	0.162	0.2	0.0754	0.377	0.2	0.0576	0.288	0.2	0.0478	0.239
0.3	0.0528	0.176	0.3	0.117	0.389	0.3	0.0852	0.284	0.3	0.0750	0.250
0.5	0.113	0.225	0.5	0.200	0.400	0.5	0.152	0.304	0.5	0.138	0.275

TABLE I (*Concluded*)

C_a	Cu_b	Cu_b/C_a
DL-Lysine Cu_f 0.0180 (1.15 μg per ml) *P* 0.390; pH 6.704		
0.2	0.0552	0.276
0.3	0.0837	0.279
0.5	0.140	0.279
DL-Histidine Cu_f 0.0250 (1.59 μg per ml) *P* 0.775; pH 6.406		
0.02	0.0167	0.836
0.05	0.0458	0.915
0.1	0.0893	0.893
0.3	0.267	0.889
0.5	0.435	0.870
Histamine Cu_f 0.0182 (1.16 μg per ml) *P* 0.400; pH 6.693		
0.1	0.0902	0.902
0.2	0.188	0.940
0.3	0.276	0.919
5.0	4.325	0.865
Pentaglycine Cu_f 0.0179 (1.14 μg per ml) *P* 0.388; pH 6.706		
0.3	0.0249	0.0830
0.5	0.040	0.0879
1.0	0.0942	0.0942
2.0	0.193	0.0965

C_a	Cu_b	Cu_b/C_a
DL-Lysine Cu_f 0.0102 (0.649 μg per ml) *P* 0.0485; pH 7.610		
0.3	0.0936	0.312
0.5	0.168	0.336
DL-Histidine Cu_f 0.0180 (1.15 μg per ml) *P* 0.390; pH 6.704		
0.3	0.250	0.833
0.5	0.407	0.813
Glycylglycine Cu_f* 0.0235 (1.50 μg per ml) *P* 0.388; pH 6.706		
0.1	0.0678	0.678
0.2	0.144	0.721
0.3	0.216	0.721
0.5	0.356	0.712

C_a	Cu_b	Cu_b/C_a
DL-Lysine Cu_f 0.0263 (1.68 μg per ml) *P* 0.0485; pH 8.280 HCO_3^- 0.18 *M* (NaCl absent)		
0.5	0.188	0.376
DL-Serine Cu_f 0.0179 (1.4 μg per ml) *P* 0.388; pH 6.706		
0.2	0.0632	0.316
0.3	0.100	0.334
0.5	0.184	0.367
Diglycylglycine Cu_f 0.0180 (1.15 μg per ml) *P* 0.390; pH 6.704		
0.3	0.0579	0.193
0.5	0.0920	0.184
1.5	0.258	0.172
2.5	0.432	0.173

C_a	Cu_b	Cu_b/C_a
DL-Histidine Cu_f 0.0263 (1.68 μg per ml) *P* 0.0485; pH 8.280 HCO_3^- 0.18 *M* (NaCl absent)		
0.5	0.620	1.24
Imidazole Cu_f 0.0182 (1.16 μg per ml) *P* 0.400; pH 6.693		
0.3	0.0231	0.077
0.5	0.0435	0.087
1.0	0.109	0.109
Tetraglycine Cu_f 0.0179 (1.14 μg per ml) *P* 0.388; pH 6.706		
0.3	0.0327	0.109
0.5	0.0550	0.110
1.0	0.114	0.114
2.0	0.240	0.120

NOTE: The meaning of the symbols employed in this and following tables is:
C_a = initial concentration of amino acid (ligand) in millimoles per liter.
Cu_b = concentration of bound copper in milliatoms per liter.
Cu_b/C_a = atoms of copper bound by one molecule of amino acid (ligand).
Cu_f = concentration of free copper in the solutions in milliatoms per liter.
P = partial pressure of CO_2 in the equilibrated gas mixture.
pH = calculated final pH of the solution.

Related Compounds

Similar experiments were carried out with a number of compounds that possess a structure in some way related to the structure of the amino acids. These were: acetic acid and acetate (pH 4–5 and pH 6–7), DL-α-alanine ethyl ester, ethanolamine, hippuric acid, 2,5-diketopiperazine, and 5-amino-*n*-caproic acid. The results obtained with these substances will be discussed later.

Proteins

The results obtained with three crystallized proteins are set out in Table II. Ovalbumen proved to be too sensitive to surface denaturation for this type of

TABLE II

The combination of copper with proteins

C_a	Cu_b	Cu_b/C_a	C_a	Cu_b	Cu_b/C_a	C_a	Cu_b	Cu_b/C_a
Bovine plasma albumen (M.W. 70,000) Cu_f 0.0185 (1.17 μg per ml) P 0.410; pH 6.682			Pepsin (M.W. 36,000) Cu_f 0.0182 (1.16 μg per ml) P 0.400; pH 6.693			Lysozyme (M.W. 13,900) Cu_f 0.0182 (1.16 μg per ml) P 0.400; pH 6.693		
0.005	0.0259	5.18	0.005	0.0216	4.31	0.5	0.0195	0.0389
0.05	0.255	5.10	0.01	0.0445	4.45	1.0	0.0391	0.0391
0.10	0.507	5.07	0.02	0.0942	4.71			
			0.05	0.237	4.74			
			0.10	0.446	4.46			

investigation, and work on this protein was as a consequence abandoned. The investigations with proteins were extended to include the effect of dialyzing a solution of the copper–protein obtained in the usual manner so that a number of successive equilibrium states were established. Dialysis was against a solution containing the same concentration of sodium bicarbonate and chloride, and an attempt was made to keep both the dialyzed solution and the dialyzate in equilibrium with the same partial pressure of carbon dioxide. The concentration of total copper both inside and outside the sac was measured at each equilibrium. The Donnan effect at the ionic strengths employed was considered to be negligible (40). Equilibrium at each stage was usually attained in approximately 12 hours. The results of the dialysis experiments for bovine plasma albumen are given in Table III. Similar

TABLE III

The equilibrium between free copper and copper bound by bovine plasma albumen as determined by dialysis at 25° C

Cu_b	Cu_f	Cu_b/C_a
0.256	0.0185	5.10
0.221	0.0137	4.40
0.200	0.00744	4.00
0.177	0.00567	3.53
0.169	0.00383	3.37
0.159	0.00235	3.17
0.151	0.00104	3.01
0.150	0.0000	3.00

NOTE: $[HCO_3^-]$: 0.04 M; [NaCl] : 0.20 M; P : 0.410; C_a : 0.05; pH : 6.682.

dialysis experiments were made with pepsin, but unfortunately during the lengthy period required for successive equilibrations, a certain amount of protein breakdown occurs (see also (41)).

Manometric and Chemical Investigation of the Combination of Copper with Amino Acids

When copper reacts with solutions of DL-methionine, an insoluble complex is formed. The precipitate has been isolated and purified; analysis for Cu and N showed it to be Cu(methionine)$_2$. A suspension of cystine will also react with the copper supplied by malachite to form an insoluble complex. Analysis of this compound reveals that it differs from most of the other amino acid complexes in containing only one atom of copper per molecule of cystine.

Manometric Determinations

In these calculations the volume of 1 gram molecule of carbon dioxide (N.T.P.) was taken, following van Slyke (42), as 22.26 liters. As is customary, the volumes of carbon dioxide absorbed or evolved at 25° C are reduced to N.T.P.

TABLE IV

Chemical determination of copper and the liberation of CO_2 for the interaction of copper and amino acids at 25° C

Final pH	Final HCO_3 (mmoles)	μl CO_2 liberated per ml soln.	Bound copper (mmoles)	μl CO_2 equiv. to Cu_b per ml	Cu_b/C_a
		Glycine : 0.025 *M*			
5.657	8.79	49.3	12.0	133.7	0.481
5.814	12.7	67.8	11.4	127.0	0.456
6.013	16.5	93.1	11.3	125.9	0.452
6.324	41.1	100.4	11.3	125.9	0.452
6.639	5.35	116.9	11.2	124.7	0.448
6.909	10.07	123.2	11.2	124.7	0.448
7.506	40.0	126.0	11.3	125.9	0.452
7.928	100.5	117.0	11.4	127.0	0.456
8.127	150.7	112.5	11.5	128.1	0.460
8.277	200.9	109.5	11.6	129.2	0.464
		DL-α-Alanine : 0.025 *M*			
5.675	9.16	37.0	11.6	129.3	0.464
5.826	13.0	57.0	11.1	123.7	0.444
6.035	17.3	70.0	10.9	121.4	0.436
6.328	41.5	87.5	10.9	121.4	0.436
6.672	5.67	106.5	10.9	121.4	0.436
6.931	10.4	113.0	10.9	121.4	0.436
7.514	40.4	117.5	11.1	123.7	0.444
7.913	100.0	113.0	11.0	123.7	0.440
8.098	150.5	111.5	11.0	123.7	0.440
8.235	200.7	106.0	11.0	123.7	0.440
		DL-Lysine : 0.025 *M*			
5.650	8.65	34.5	10.4	115.8	0.416
6.094	16.5	75.0	9.7	108.0	0.388
6.664	5.49	95.0	9.5	105.8	0.380
7.526	40.1	100.0	9.2	100.5	0.368
7.974	100.2	94.0	8.9	99.2	0.356
8.277	200.3	91.0	8.8	98.0	0.352

At the pH of the solutions employed, the amino acid exists almost entirely in the form of the zwitterion. The formation of a complex of the undissociated CuR_2 type can therefore be conveniently represented by the following equation in the case of glycine.

[1] $$4\ \overset{+}{N}H_3.CH_2C\overset{-}{O}O + CuCO_3.Cu(OH)_2 \rightarrow 2(NH_2.CH_2.COO)_2Cu + CO_2 + 3H_2O$$

Every atom of copper reacting with two molecules of amino acid should therefore lead to the liberation of 0.5 molecules of carbon dioxide when the observed evolution is corrected for retention.

The results obtained for glycine, DL-α-alanine, and DL-lysine are brought together in Table IV. The pH values are calculated for the conditions existing at the completion of the reaction. The third column of Table IV gives the observed volume of carbon dioxide liberated per ml of solution after correction for retention; the value of a blank for similar solutions not containing the amino acid has also been subtracted. The amount of copper bound to the amino acid in the solutions is known from the results of the chemical analyses (28, 29) and is also given in Table IV. The fifth column of Table IV shows the volume of carbon dioxide to be expected from this experimentally determined concentration of bound copper on the basis of equation [1].

The results obtained with DL-α-alanine are shown in Fig. 1. The reaction of malachite with DL-methionine is shown in Fig. 2, and with cystine and α,δ-diaminoadipic acid in Fig. 3.

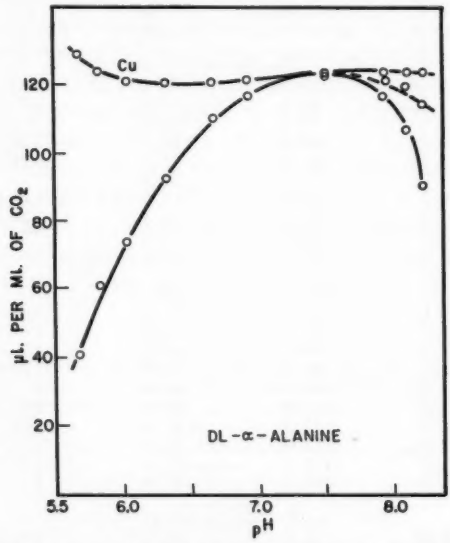
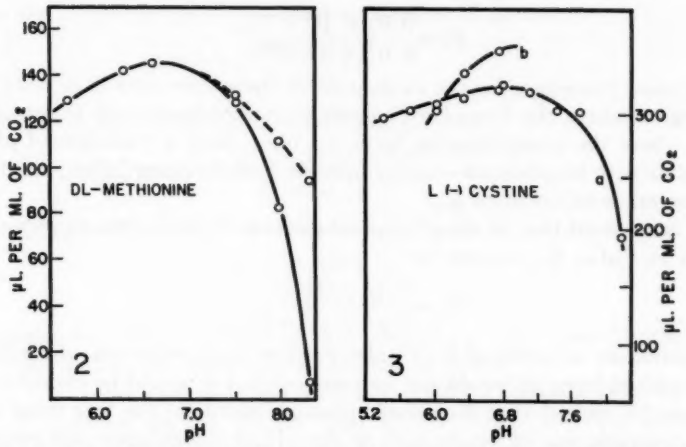


FIG. 1. The effect of pH on the amount of copper bound and the volume of carbon dioxide liberated by the reaction of malachite with DL-α-alanine at 25° C. "Cu" represents the theoretical amount of evolved carbon dioxide equivalent to the experimentally determined complexed copper in the solution, on the basis of 1 atom of copper ≡ 0.5 moles of carbon dioxide. —— Actual volume of carbon dioxide evolved. - - - Actual volume of carbon dioxide evolved, corrected for retention.



FIGS. 2 and 3. The effect of pH on the volume of carbon dioxide liberated by the reaction of malachite at 25° C with:
(Fig. 2) DL-methionine;
—— Actual volume of carbon dioxide evolved; - - - actual volume of carbon dioxide evolved, corrected for retention.
(Fig. 3) (*a*) L-cystine; (*b*) α,δ-diaminoadipic acid.

The reaction of malachite with DL-cysteine was also investigated. The extreme susceptibility of this compound to oxidation in the presence of dissolved copper made it essential to exclude oxygen rigorously from the Warburg vessel. The results indicated that the reaction of copper and DL-cysteine gave rise to a complex of the 2:1 type i.e. CuR_2, but the system was a labile one at the pH values employed because a little of a black precipitate, presumably cupric sulphide, sometimes appeared.

The Dissociation Constant of the Copper–Glycine Complex

The equilibrium between cupric ion and glycine is often represented as a stability constant K_c where K_c is given by equation [2].

$$[2] \qquad K_c = \frac{[CuG_2]\ [H^+]^2}{[Cu^{++}]\ [G^-H^+]^2}$$

It is only in recent years that attempts have been made to evaluate the separate constants, K_1 and K_2, of equations [3] and [4], and still more recently, to obtain them in the form of thermodynamic dissociation constants. It will be seen that;

$$K_c = K_1.K_2$$

where,

$$[3] \qquad K_1 = \frac{[Cu^+G]\ [H^+]}{[Cu^{++}]\ [G^-H^+]}$$

and

[4] $$K_2=\frac{[CuG_2]\ [H^+]}{[Cu^+G]\ [G^-H^+]}.$$

The usual procedure for the evaluation of these constants is to work in an acid region where the form Cu^+G tends to predominate, and in an alkaline region where the predominating form is CuG_2. Such a variation of pH was impossible with bicarbonate – carbon dioxide buffers; nevertheless, an attempt has been made to calculate K_c.

If it is assumed that in the glycine solutions of Table I the complex existed entirely as CuG_2, K_c is given by:

[5] $$K_c=\frac{[Cu_b]\ \ [H^+]^2}{[Cu^{++}]\ \ [C_a-2Cu_b]^2}.$$

If significant amounts of Cu^+G also exist in these solutions, the values of K_c calculated from [5] would not be constant but it would be expected from equations [3] and [4] that K_c would approach nearer to the true value as the initial concentration of amino acid or the pH of the solution was increased. The values of pK_c calculated by means of equation [5], except that activities of cupric and hydrogen ions were used in place of concentrations, are set out in Table V. It will be seen that the trend of the values, as the pH and initial

TABLE V

The calculated values of pK_c for the copper–glycine complex at 25° C

Initial concn. of glycine (mmoles)	pK_c at pH values of:				
	6.309	6.682	6.730	7.008	7.610
5	3.56				
1			3.62		
0.5	3.19	3.64	3.43	3.58	3.70
0.4		3.55	3.36		
0.3	3.11	3.51	3.36	3.63	3.67
0.25			3.32		
0.20	3.10	3.75	3.23	3.66	3.60
0.10	2.66	3.41	3.37	3.51	3.56
0.05		2.94	2.54		

concentration of glycine increase, is towards a value of pK_c in the neighborhood of 3.7 at 25° C and pH 7.610. This value may be compared with the value of 3.58 given by Keefer (11) and with those given by Albert (12) and Monk (13). The latter workers expressed the equilibrium in the following manner, copper being represented as combining with the anion of the amino acid, rather than the zwitterion:

[6] $$K_1=\frac{(Cu^{++})\ (G^-)}{(Cu^+G)} \text{ and } K_2=\frac{(Cu^+G)\ (G^-)}{(CuG_2)},$$

[7] $$K_c=K_1.K_2=\frac{(Cu^{++})\ (G^-)^2}{(CuG_2)}.$$

The value of pK_c given by Albert for copper–glycine at 20° C is 15.40 and the value of $pK_1.K_2$ given by Monk where K_1 and K_2 are thermodynamic constants, is 15.58 at 25° C.

From the thermodynamic dissociation constant of glycine

$$[8] \qquad 10^{-9.886} = \frac{(H^+)\ (G^-)}{(G^-H^+)},$$

it is possible to transform $K_1.K_2$ of equation [7] into:

$$[9] \qquad K_1.K_2 = \frac{(Cu^{++})\ (G^-H^+)^2 \times 10^{-19.772}}{(CuG_2)\ (H^+)^2}.$$

Taking the value of pK_c from the results of Table V, as 3.70 where:

$$[10] \qquad 10^{-3.7} = \frac{(CuG_2)\ (H^+)^2}{(Cu^{++})\ (G^-H^+)^2}$$

and substituting [10] in [9], the present results give the value for $pK_1.K_2$ of 16.07, expressed in the same manner as that of Monk.

When it is considered that the values of Table V indicate that the value of pK_c is probably slightly greater than 3.7 (i.e., that $pK_1.K_2$ is rather less than 16.07), the agreement with the value given by Monk (15.58) seems satisfactory. The value lies, in fact, within the range of variation of recently reported values of the constant obtained by very different methods (27).

Discussion

The general technique of equilibrating the system with the sparingly soluble copper salt malachite has made it possible to investigate the combination of copper with amino acids and other compounds.

The combination of glycine with copper has been studied over a range of pH and amino acid concentration. The other amino acids have not been investigated in such detail. Nevertheless, as the concentration of free copper in the solutions was defined by the solubility of malachite, the results afford a convenient means of comparing the relative avidity of the different compounds for copper. The essential correctness of this assumption is shown in the calculation of the dissociation constant of the glycine–copper complex. If the free copper observed in these solutions had been assumed to be entirely in the form of Cu^{++} a very different value for the dissociation constant would have been obtained.

The ratio of the number of amino acid molecules bound to one atom of copper usually approached 2:1 (i.e. $Cu_b/C_a = 0.5$) when the initial concentration of the amino acids was in the neighborhood of 0.025 *M*. Hence the large deficit in the volume of evolved carbon dioxide, noticed in the manometric experiments at the more acid pH values, cannot be attributed to the formation of a different type of complex at these pH values. The most likely explanation is possibly the dissociation of the neutral complex in the following manner:

$$[11] \qquad 2\ [(NH_2.R.COO)_2Cu] + 4H_2O + 4CO_2 \rightleftarrows 2[(N^+H_3.R.COO)_2Cu] + 4HCO_3^-.$$

According to [11] an increase in bicarbonate ion concentration should occur in the solution. No such increase in the reaction is depicted in equation [1]. Experimental determinations have confirmed that an increase in bicarbonate

ion concentration has occurred in these solutions, over and above the increase to be expected from the reduced evolution of carbon dioxide. It is interesting that the fall in liberated carbon dioxide as the pH decreases below the point of maximum evolution, is much less marked in the case of DL-methionine (Fig. 2) and cystine (Fig. 3). This is as it should be since the insoluble fraction of the partly soluble complexes formed in these two cases is in the neutral state.

At the lower concentrations of amino acids (of the order of 5×10^{-4} *M*) the complexes were appreciably dissociated (i.e. $Cu_b/C_a < 0.5$); the free amino acid, the complex CuR_2, and possibly the complex Cu^+R all being present in the solution in appreciable amounts.

The results also show that the higher the pH of the solution, the more stable is the complex. This might not be evident from a first glance at Table I; both glycine and DL-lysine, for example, appear to bind more copper at pH 6.309 than at pH 7.610. It must be remembered, however, that malachite is more soluble at the lower pH and consequently the equilibrium concentration of cupric ion is greater. When the results are expressed in terms of Cu_b/Cu^{++} the marked effect of pH is clearly seen as shown in Table VI. It will be seen that the second amino group of lysine confers no additional power to bind copper.

TABLE VI

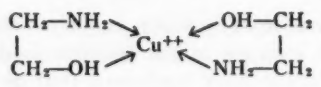
Effect of pH on the binding of copper by glycine and DL-lysine

	Cu_b/Cu^{++}*	
pH	Glycine	Lysine
6.309	97	84.5
7.610	8400	8370

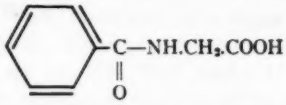
*Concentration of amino acid: 5×10^{-4} *M*.

Experiments with acetate ions (pH 6–7) revealed that these were practically incapable of binding copper, although it was easy to demonstrate that at lower pH values (4–5) complexes were formed as noticed by other workers (43). The great stability conferred on the complex by an α-amino group is thus clearly demonstrated, as is the fact that the greatest stability is found when the amino group is adjacent to the carboxyl group. For example α-alanine is able to bind nearly twenty times more copper than β-alanine under comparable conditions (pH 6.706). In the ϵ-position, as in 5-amino-*n*-caproic acid, the ability to bind copper is entirely lost.

Ethylenediamine is known to form very stable complexes of the bidentate type with copper. Ethanolamine, however, was only able to bind negligible amounts of copper, so that the co-ordinating power of the hydroxyl group is much less than that of the carboxyl group.

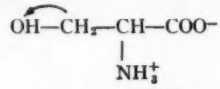


The behavior of compounds containing the favorable α-NH_2 : COOH grouping is considerably modified by the presence of substituents in the molecule. Thus it was found that hippuric acid was unable to bind copper.

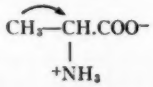


Likewise the esterification of the carboxyl group as in DL-α-alanine ethyl ester eliminated the copper complexing powers of the acid.

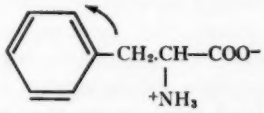
DL-Serine forms the normal 2:1 complex with copper but it is more stable than that formed with DL-α-alanine. This may be attributed to the electrostatic inductive effect of the OH group, which is also reflected in the lower pK_a value of DL-serine compared with that for DL-α-alanine.



The manometric results also indicated that DL-cysteine formed a more stable complex than DL-α-alanine, presumably for the same reason. The substitution of an α-methyl group in glycine to give α-alanine, on the other hand, had an opposite inductive effect to that exerted by SH or OH.



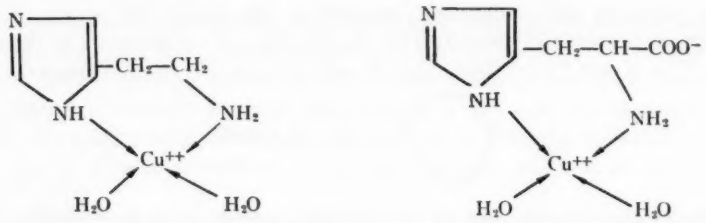
The substitution of the benzyl group might be expected to enhance the stability of the complex, and this in fact was observed for DL-β-phenylalanine.



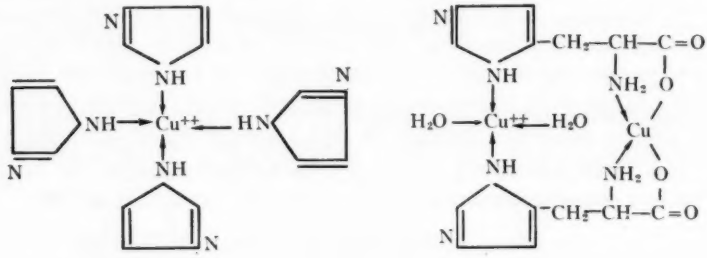
Optical stereoisomerism, as reflected in the behavior of DL-glutamic acid and L-glutamic acid, does not seem to have much influence on complex formation. The slightly greater stability of the glutamic acid complexes as compared with that of the α-alanine complex could be attributed to the stabilizing influence of the second carboxyl group of glutamic acid acting, in this respect, in the opposite sense to the second $-^+NH_3$ group of lysine, which exerts a repulsive or weakening action.

DL-Histidine presents a more complicated behavior. In acid or neutral solutions, the results suggest that the chief complex is a 1:1 ratio type. At pH 8.2 the ratio of Cu_b/C_a is (1.24), which suggests that a complex of the form Cu_3(histidine)$_2$ may also be present in alkaline solutions ($Cu_b/C_a = 1.5$).

Histamine apparently forms a 1:1 complex with copper. As there are no carboxyl groups in the molecule, the two nitrogen atoms must be involved. This suggests that a proportion of the copper bound to histidine might also form part of an analogous structure:

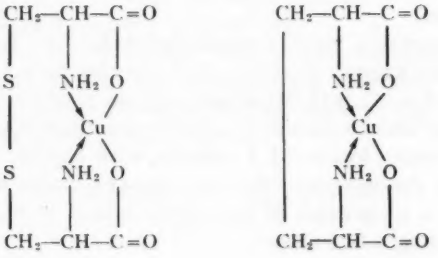


Tetra co-ordination is represented as being maintained by the binding of water molecules, a fact already known (25, 44). The binding of copper by imidazole suggests an alternative formulation for the 1:1 histidine complex. Confirmatory findings for imidazole have been reported (18, 19).



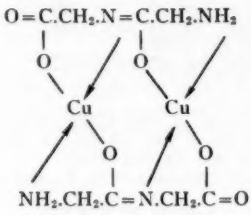
Another compound of similar nature is DL-tryptophane, which forms a 2:1 complex. The co-ordinating power of the indole ring nitrogen atom of tryptophane is thus less than that of the nitrogen in the iminazole ring of histidine. In fact, it is possible to predict qualitatively the co-ordinating tendency of these secondary nitrogen groups from their basicities (See also (24).) Thus indole, the parent compound of tryptophane, is only weakly basic whereas imidazole, the parent of histidine and histamine, is strongly basic, and these two compounds therefore bind more copper than tryptophane. These observations can be extended to other ring systems. Thus despite the absence of an α-NH_2 group, proline can bind approximately two-thirds of the copper that glycine can, which is in keeping with the strongly basic nature of pyrrolidine.

It is interesting to find that α,∂-diaminoadipic acid combines with copper in a similar manner to that of cystine (i.e. 1:1) except that the complex is



soluble. Presumably, stable internal compounds of the form shown below are formed.

Under comparable conditions the peptides of glycine show anomalous behavior. Thus, if the binding of copper by a molecule of glycine is represented by 0.5, the corresponding values per molecule of peptide in the series from diglycine to pentaglycine are 1.1, 0.35, 0.21, and 0.17 respectively. There is no evidence, therefore, of any significant binding of copper by the peptide linkage as such (see also (23)); this conclusion is supported by the inability of either 2,5-diketopiperazine or hippuric acid to combine with copper. The peptide bond can undergo keto–enol tautomerism (45), and the exceptionally strong binding power of glycylglycine may reside in this shift.



The behavior of the three proteins studied is in sharp contrast to that of amino acids at similar molar concentrations, and in the presence of similar concentrations of cupric ions. Whereas the amino acid complexes are dissociated to an appreciable extent, and the extent is dependent on concentration, the ratio Cu_b/C_a for bovine plasma albumen and pepsin at a constant concentration of cupric ions remains sensibly constant over a 20-fold range of protein concentration. These ratios correspond approximately to the binding of five atoms of copper to every molecule of albumen and 4.5 atoms to every molecule of pepsin. It seems justifiable to conclude that copper is bound to these proteins at a number of specialized sites, and these are independent of the sites on other molecules. Measurements at higher concentrations of total copper than these provided by malachite would be necessary to obtain some idea of the total number of sites in the molecule that are able to bind with copper. (See also (26).) Lysozyme differs markedly from the other two proteins in its relative lack of ability to bind copper; the relatively large number of $-\overset{+}{N}H_3$ groups provided by the lysine residues are the most probable explanation for the instability of the complex.

It has been shown by means of equilibrium dialysis that a reduction in the concentration of free copper reduces the number of atoms bound by albumen and pepsin. The quantitative results suggest that the sites are not identical. Bovine plasma albumen retains three atoms of copper, when the concentration of free copper is vanishingly small, and pepsin appears to retain one atom under similar conditions.

Acknowledgments

I wish to record my appreciation for the guidance and encouragement of Dr. J. Brooks of this laboratory, during the course of this work.

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THE CATALYSIS OF ASCORBIC ACID OXIDATION BY COPPER AND ITS COMPLEXES WITH AMINO ACIDS, PEPTIDES, AND PROTEINS[1]

J. F. SCAIFE[2]

Abstract

The oxidation of solutions of ascorbic acid catalyzed by copper and the copper complexes of amino acids, peptides, and proteins has been investigated by manometric and chemical means. The copper-containing solutions were prepared by equilibration with the sparingly soluble salt malachite. The catalytic activity of the complexed copper was found to be dependent upon the type of complexing molecule. For amino acids the more firmly bound copper atoms tended to be the best catalysts. The catalytic activity of the copper complexed to several glycine peptides was approximately one half of the mean value of that of the copper complexed to amino acids. The catalytic activity of copper complexed to bovine plasma albumen and pepsin was lower than that of copper complexed with amino acids or peptides. The more firmly bound copper atoms on pepsin showed a higher catalytic activity than the mean value for all the complexed copper atoms. Sodium chloride markedly reduced the catalytic activity of both free copper and complexed copper. In the absence of sodium chloride free copper was 10 times as effective a catalyst as copper complexed to glycine. In the presence of 0.178 *M* sodium chloride the complexed copper was twice as effective as free copper.

Introduction

The sensitivity of ascorbic acid (AsA) to the catalytic effects of minute traces of copper is well known. Consequently the catalyzed oxidation of ascorbic acid provides a suitable system for observing the effects of copper-complexing compounds upon the catalytic activity of this metal. In the case of copper–protein complexes, a further comparison may be made with the copper of the naturally occurring enzyme ascorbic acid oxidase.

One of the main difficulties in such a study is to determine the amount of free copper in solution in equilibrium with any complex that may be present. This is especially important in the case of AsA oxidation as the catalytic activity of free copper is very high. Moreover, certain buffer constituents, like bicarbonate and phosphate, can form sparingly soluble salts with copper and so reduce the effective concentration in solution. Still other buffers like citrate and acetate can themselves form complexes with copper. In the present work the solutions have been equilibrated with the sparingly soluble salt malachite in the presence of bicarbonate buffer. By this means the amount of free copper in solution is always determined by the solubility product of the malachite under the prevailing conditions, irrespective of the presence or absence of a complexing compound. It is thus possible to evaluate the catalytic activity of the free copper alone, and in a separate experiment, the catalytic activity of the mixture containing free copper and copper complex.

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The oxidation of ascorbic acid in solution is very much dependent upon the conditions. Under alkaline conditions a non-catalytic autoxidation can take place (1, 2) but in neutral or acid solutions no oxidation occurs in the absence of copper (3–5). The first stage of the oxidation is reversible to produce the lactone, dehydroascorbic acid (DHA) (6–8). This compound breaks down with increasing rapidity above pH 4.0, to give the strong acid, 2,3-diketogulonic acid (DKG) (9–11). This irreversible change is independent of the presence of copper (12, 13). Subsequent changes give rise to threonic and finally oxalic acids (14, 15). The copper catalyst undergoes a cyclic reduction and oxidation and hydrogen peroxide appears to be formed during the reaction (4, 9, 16). Depending upon the conditions, the hydrogen peroxide can react in a variety of ways, either by decomposition or directly with ascorbic or dehydroascorbic acids (17). The net result is that for each molecule of ascorbic acid oxidized the amount of oxygen consumed can vary from one to two atoms per molecule of AsA. For the enzyme-catalyzed reaction the ratio is always unity.

A considerable number of factors influence the course of the reaction. First, the reaction is light sensitive (18, 19) and its rate is also dependent upon the pH (4, 20) and the partial pressure of oxygen (21). Second, any compound which can alter the concentration or nature of the copper in solution will have a profound effect upon the rate of the catalyzed reaction. Thus the inhibitory nature of amino acids (22–25) and proteins (26) has been noticed, as has that of certain buffers (20, 27, 28) by reason of their complexing powers with copper. Reducing compounds like glutathione (3) and glucose (29) also affect the rate of reaction. Lastly, a large number of compounds have been shown to exert an influence on the catalyzed oxidation of AsA, sulphur-containing compounds (30–32), sugars (20, 33, 34), and inorganic salts (26, 34–37), notably chlorides (5, 23, 34, 38), being particularly effective.

Methods and Materials

A previous paper (39) has described the preparation of solutions containing copper complexes and free copper by equilibration with malachite. It was hoped that it would be possible to determine the oxidation of AsA in such solutions in the presence of solid malachite. It was found, however, that the oxidation of AsA was rapidly catalyzed on the surface of the malachite particles. Consequently these were separated from the equilibrated buffer solutions by means of fine porosity filter sticks. The procedure finally adopted, therefore, was to mix one volume of AsA solution (adjusted to the final pH of the buffered mixture), with eight volumes of the filtered solution that had previously been equilibrated with malachite. Unless otherwise stated, all concentrations and pH values quoted in the present work are those prevailing after dilution from eight to nine volumes, and the same gas mixtures were used for equilibration and oxidation. All the gas mixtures consisted of 20% oxygen and various proportions of carbon dioxide and nitrogen. Unless otherwise stated, all experiments were made at 25° C in a room illuminated with a red photographic safe light, and the gas mixtures were saturated with water vapor at this temperature before being passed through the reaction vessels.

Glassware that had been cleaned in hot acid dichromate solutions could not be used after drying at 100° C, regardless of the amount of water used to wash it beforehand. Oxidations carried out in these vessels were markedly reduced in rate. Apparently, a firmly adhering layer of some chromium compound is rendered soluble by heating at 100° C. All apparatus was cleaned by treatment with alcohol and then nitric acid.

The course of the oxidation of AsA was followed by direct titration, and gasometrically in parallel experiments, by means of the Warburg technique.

The Determination of the Rate of Oxidation by Titration

(*a*) *The Reaction Vessel*

This was identical with that previously described (40) except that a side arm, like that of a Warburg vessel, was attached near one end of the main compartment. It was covered completely with aluminium foil, to exclude stray illumination. The main compartment contained 24 ml of the equilibrated buffer solution while the side arm held 3 ml of AsA solution. The vessel, immersed in the thermostat, was shaken rapidly to and fro and, after equilibration with the gas mixture, the contents were mixed. Samples were withdrawn at appropriate intervals and all experiments were done in duplicate.

(*b*) *Titration*

The method was essentially that of Tillmans (41). Oxidation in a 2-ml sample drawn from the reaction vessel was stopped by mixing it with 8-ml of a 4% solution of metaphosphoric acid (35). This solution was found to be stable in the dark, despite the presence of the various catalysts. Direct titration with 2,6-dichlorophenol indophenol did not give a good end point, but a sharp one was obtained when a 2-ml aliquot of this mixture was diluted with 5 ml of the metaphosphoric acid solution. The indophenol solution contained 0.5 g of the dye in 0.5% sodium bicarbonate solution and was stored at 0° C. Approximately 1 ml of this solution was equivalent to 0.75×10^{-6} gram moles of AsA.

The Manometric Determination of the Rate of Oxidation

If for no other reason than that provided by the formation of the strong acid, DKG, from DHA, it would be expected that oxidation would be accompanied by a decrease in the pH of the buffered solution, and hence an evolution of carbon dioxide which would tend to obscure the fall in pressure in the Warburg vessels due to an oxygen absorption. An attempt has been made to measure the separate rates of both reactions by means of the "indirect method" of Warburg (42).

In the present instance, one vessel contained initially 1 ml of buffer in the main chamber and 0.125 ml of AsA solution in the side arm; in the second vessel the corresponding volumes of the same solutions were 4 ml and 0.5 ml respectively. Actually, in a given experiment each of these two vessels was duplicated. In the case of the vessels containing 4.5 ml of liquid, it was necessary to replace the Brodie solution in the manometer by a denser liquid. Acetylene tetrabromide, density 2.964, was used. It attacks rubber, however, and permeates through "tygon" tubing so that the reservoir of the mano-

meters was made of rigid polyethylene connected by glass to an inverted rubber reservoir containing glycerine as the propellent fluid.

All chemicals used in this work were of the highest grade and were tested prior to use for the presence of copper.

Results

The Catalytic Activity of "Free" Copper

In the absence of amino acids or other complexing molecules the total concentration of "free" copper in an equilibrated buffer solution is the sum of the concentrations of Cu^{++} and $CuCO_3$ complex (40). Ascorbic acid dissolved in control solutions did not oxidize at an appreciable rate in the absence of an added catalyst.

(*a*) *The Effect of Copper Concentration*

Malachite was equilibrated with various concentrations of bicarbonate and partial pressures of carbon dioxide at 25° C. The filtered solutions were analyzed for total copper (43) and diluted to the same concentration of bicarbonate. The same gas mixture was used in every case during the oxidation at 25° C; the solutions being tested were consequently of the same pH but contained different concentrations of free copper. The pH chosen for these experiments was 6.155. Some of the manometric and titrimetric results are shown in Figs. 1 and 2.

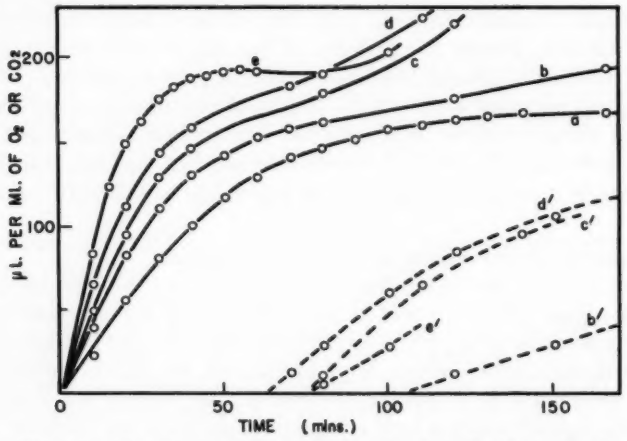

FIG. 1. The absorption of oxygen and evolution of carbon dioxide during the oxidation of ascorbic acid at 25° C in the presence of dissolved malachite.

O_2 absorption curves	Initial HCO_3^-, (*M*)	Initial CO_2, (atm)	Final concn. copper (μg per ml)
a	0.08	0.0484	0.0613
b	0.08	0.388	0.102
c	0.04	0.388	0.186
d	0.02	0.388	0.342
e	0.01	0.388	0.602

The corresponding curves for the evolution of CO_2 are *a'*–*e'*.

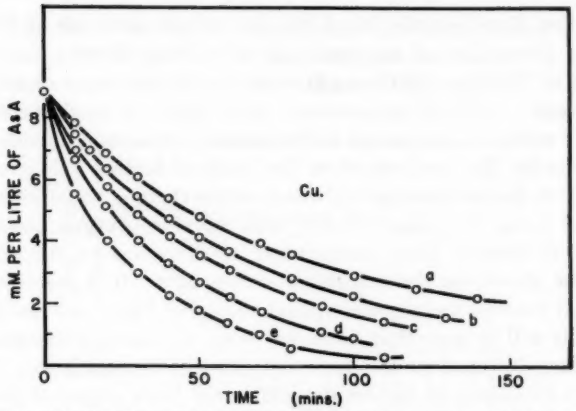

FIG. 2. The oxidation of ascorbic acid at 25° C in the presence of dissolved malachite.

Disappearance of ascorbic acid

	Initial HCO_3^- (M)	Initial CO_2 (atm)	Final concn. copper (μg per ml)
a	0.08	0.0484	0.0613
b	0.08	0.388	0.102
c	0.04	0.388	0.186
d	0.02	0.388	0.342
e	0.01	0.388	0.602

It will be seen from Fig. 2 that an increase in the concentration of free copper increases the rate of disappearance of AsA. At a given concentration of copper, the rate diminishes progressively with time, and follows essentially first-order kinetics. As is shown in Table I for low concentrations of copper, the catalytic power per unit of free copper (b/a) is high, but with increasing concentrations of copper the catalytic activity decreases.

TABLE I

The effect of the concentration of free copper on the rate of disappearance of ascorbic acid and oxygen

Free copper, μg per ml *a*	$-\Delta AsA/\Delta t$, mmole/liter min *b*	b/a	$-\Delta O_2/\Delta t$, mmole/liter min *c*	c/a
0.061	0.097	158	0.122	199
0.084	0.107	128	0.170	204
0.102	0.138	135	0.180	176
0.141	0.148	105	0.211	150
0.186	0.212	114	0.218	117
0.276	0.196	71	0.233	85
0.342	0.228	67	0.283	83
0.602	0.354	59	0.375	62

NOTE: Concentration of AsA : 8.88 mmole per liter; P : 0.388 atm CO_2; $[HCO_3^-]$: 0.00889 M; pH : 6.155.

It is more difficult to interpret the results of the manometric measurements. The dissociation constants of AsA, DHA, and DKG suggest that in a bicar-

bonate – carbon dioxide buffer the formation of one molecule of DHA would result in the absorption of one molecule of carbon dioxide, and that the mutarotation of DHA to DKG would result in the evolution of one molecule of carbon dioxide. If DHA mutarotation were rapid, as would be expected at this pH, there would be no change in the pressure of carbon dioxide, and then any carbon dioxide liberated would be the result of further oxidative changes. Unfortunately in the initial stages of the reaction there is some uncertainty in the calculated value of γ (i.e. CO_2/O_2) and therefore oxygen absorption has been calculated directly from manometric readings up to the point where reliance can be placed on the calculated values of γ. It is possible therefore that the initial rates of oxygen absorption shown in Fig. 1 are too high, since from Table I it will be seen that, mole for mole, the rates of disappearance of AsA are, in fact, lower than the apparent rates of disappearance of oxygen. There is a net evolution of carbon dioxide in the later stages of the reaction which is tentatively attributed to the oxidative changes occurring after the formation of DKG.

(*b*) *The Effect of pH and Ascorbic Acid Concentration on the Rate of Oxygen Absorption*

To investigate the effect of pH, malachite was equilibrated with two solutions of bicarbonate in the presence of 0.05 atm of carbon dioxide. The filtered solutions were then used to oxidize AsA in the presence of 0.05, 0.2, and 0.4 atm of carbon dioxide, thus varying the pH without an alteration in the concentration of copper. The results are set out in Table II. There is a decrease in the rate of oxygen absorption in going from pH 6.44 to 7.65.

TABLE II

The effect of pH on the rate of absorption of oxygen by ascorbic acid

Free copper, μg per ml	P, atm CO_2	$[HCO_3^-]$, M	pH	$-\Delta O_2/\Delta t$, mmole/liter min
0.283	0.0484	0.0178	7.345	0.213
0.283	0.194	0.0178	6.742	0.263
0.283	0.388	0.0178	6.441	0.287
0.334	0.0484	0.0355	7.646	0.186
0.334	0.194	0.0355	7.043	0.245
0.334	0.388	0.0355	6.742	0.284

NOTE: Concentration of AsA : 8.88 mmole per liter.

Similar experiments were carried out in which the concentration of AsA was halved, and similar results were obtained on a reduced scale of oxygen absorption. Halving the concentration reduced the rates by approximately 40%.

(*c*) *The Effect of Sodium Chloride on the Rate of Disappearance of Ascorbic Acid*

Various amounts of sodium chloride were added to a bicarbonate–carbon dioxide buffer that had previously been equilibrated with malachite. The effect of sodium chloride upon the reaction is marked, and far outweighs the minor effects of a small change in pH caused by the alteration in ionic strength.

These results are shown in Fig. 3. As will be seen from the figure, increasing concentrations of salt not only retarded the rate of reaction, but it also altered the unimolecular course of the reaction to one of zero order.

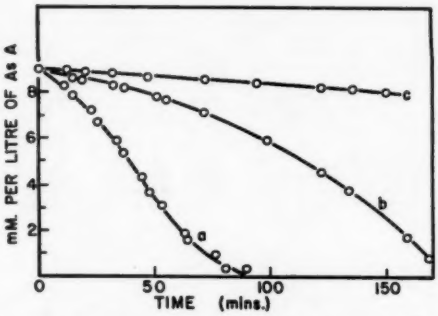

FIG. 3. Effect of sodium chloride upon the copper-catalyzed oxidation of ascorbic acid. Malachite equilibrated with 0.01 *M* $NaHCO_3$, 0.05 *M* NaCl, and *P*: 0.388 atm CO_2 to give a solution containing 2.46 μg per ml. Final concentrations in the reacting mixtures were:

Curve	HCO_3 (*M*)	NaCl (*M*)	Concn. copper (μg per ml)
a	0.00889	0.0445	1.09
b	0.00889	0.0889	1.09
c	0.00889	0.178	1.09

(*d*) *The Effect of Added Copper Sulphate on the Rate of Oxidation*

The addition of copper to a bicarbonate buffer solution should give the same results as those obtained by equilibration with malachite provided the solubility product of the basic copper carbonate was not exceeded. A few experiments of this type were made and the results are set out in Table III. The

TABLE III

The effect of copper sulphate on the rate of oxidation

Added copper, μg per ml	mmole/liter min	
	$-\Delta AsA/\Delta t$	$-\Delta O_2/\Delta t$
0.0889	0.139	0.145
0.178	—	0.196
0.445	0.271	0.279
4.45	1.16	—

NOTE: Concentration of AsA : 8.88 mmole per liter; $[HCO_3^-]$: 0.00889 *M*; pH : 6.155; *P* : 0.388 atm CO_2.

results are comparable to those of Table I when the titrimetric measurements are compared, but the rates of oxygen absorption tend to be slower than those of Table I.

TABLE IV

The catalytic activity of complexes of copper with amino acids and peptides

Cu_b	R_b	R_b/Cu_b	Cu_b	R_b	R_b/Cu_b	Cu_b	R_b	R_b/Cu_b	Cu_b	R_b	R_b/Cu_b
Glycine *R_f/Cu 0.851 P 0.775; pH 6.360			Glycine R_f/Cu 1.47 P 0.410; pH 6.636			Glycine *R_f/Cu 1.70 P 0.368; pH 6.684			Glycine R_f/Cu 2.24 P 0.194; pH 6.957		
0.0271	0.0439	1.62	0.00909	0.0198	2.18	0.0136	0.0320	2.36	0.0164	0.079	4.82
0.0537	0.111	2.07	0.0152	0.0398	2.62	0.0235	0.0705	3.00	0.0380	0.199	5.23
0.140	0.312	2.22	0.0385	0.108	2.81	0.0513	0.175	3.41	0.0675	0.265	3.93
			0.0652	0.189	2.90	0.0794	0.248	3.12	0.142	0.385	2.71
			0.0927	0.259	2.79	0.113	0.330	2.92			
			0.117	0.310	2.65	0.143	0.423	2.95			
Glycine *R_f/Cu 5.12 P 0.0485; pH 7.559			DL-α-Alanine *R_f/Cu 1.64 P 0.388; pH 6.660			β-Alanine *R_f/Cu 1.64 P 0.388; pH 6.660			DL-Glutamic acid *R_f/Cu 1.64 P 0.388; pH 6.660		
0.0217	0.135	6.22	0.0190	0.0483	2.54				0.0265	0.0462	1.75
0.0519	0.282	5.44	0.0375	0.120	3.20	0.0077	0.00898	1.17	0.0417	0.114	2.78
0.0828	0.406	4.91	0.0582	0.157	2.70	0.0134	0.0183	1.36	0.0735	0.191	2.59
0.152	0.471	3.11	0.104	0.235	2.26	0.0228	0.0433	1.90	0.123	0.267	2.18
						0.0347	0.0578	1.67	0.282	0.418	1.48
L-Glutamic acid R_f/Cu 1.53 P 0.388; pH 6.660			L-Glutamic acid *R_f/Cu 1.64 P 0.388; pH 6.660			DL-Tryptophane *R_f/Cu 1.64 P 0.388; pH 6.660			DL-Proline *R_f/Cu 1.64 P 0.388; pH 6.660		
0.0242	0.0462	1.91				0.0323	0.0753	2.33	0.0106	0.0192	1.81
0.0459	0.135	2.95				0.0680	0.226	3.32	0.0281	0.0604	2.15
0.0793	0.189	2.38	0.0857	0.200	2.34	0.105	0.330	3.14	0.0465	0.103	2.21
0.111	0.281	2.52	0.136	0.276	2.02	0.128	0.494	3.86	0.0998	0.229	2.29

TABLE IV (*Concluded*)

Cu_b	R_b	R_b/Cu_b	Cu_b	R_b	R_b/Cu_b	Cu_b	R_b	R_b/Cu_b	Cu_b	R_b	R_b/Cu_b
DL-β-Phenylalanine R_f/Cu_f 1.53 P 0.390; pH 6.658			DL-Lysine R_f/Cu_f 1.53 P 0.390; pH 6.658			DL-Lysine R_f/Cu_f 0.847 P 0.775; pH 6.360			DL-Serine R_f/Cu_f 1.53 P 0.388; pH 6.660		
0.0333	0.0885	2.66	0.0251	0.0389	1.55	0.0194	0.0284	1.46	0.0561	0.181	3.22
0.0670	0.199	2.96	0.0490	0.104	2.12	0.0424	0.0921	2.17	0.0890	0.283	3.18
0.104	0.308	2.96	0.0743	0.194	2.61	0.0666	0.141	2.12	0.163	0.388	2.38
0.178	0.321	1.80	0.132	0.306	2.31	0.122	0.235	1.92			
Imidazole R_f/Cu_f 1.51 P 0.400; pH 6.647			Glycylglycine R_f/Cu_f 1.64 P 0.388; pH 6.660			Diglycylglycine R_f/Cu_f 1.53 P 0.390; pH 6.658			Tetraglycine R_f/Cu_f 1.53 P 0.388; pH 6.660		
0.0206	0.0467	2.27	0.0248	0.0368	1.49	0.0514	0.0566	1.10	0.029	0.0404	1.39
0.0386	0.0919	2.39	0.0597	0.0890	1.49	0.0817	0.105	1.29	0.049	0.0768	1.57
0.0965	0.188	1.95	0.128	0.179	1.40	0.229	0.310	1.36	0.101	0.177	1.75
			0.192	0.268	1.40	0.384	0.519	1.35	0.214	0.360	1.69
			0.316	0.330	1.04						
Pentaglycine R_f/Cu_f 1.53 P 0.388; pH 6.660											
0.0222	0.0314	1.41									
0.0391	0.0584	1.50									
0.0838	0.140	1.67									
0.171	0.313	1.82									

NOTE: $[HCO_3^-]$: 0.0356 *M*; [NaCl] : 0.178 *M*.

The Rate of Oxidation in the Presence of Copper Complexes

(*a*) *The Rate of Disappearance of Ascorbic Acid*

In these experiments it soon became evident that the catalytic activity of the complexes was so large that the rate of oxidation at 25° C would be unmanageably rapid in their presence. Instead of lowering the temperature, the rate of the reaction was lowered by having sodium chloride present both during equilibration and oxidation. Besides the stabilizing effect on the ionic strength of the solutions, the presence of sodium chloride had the advantage that the rates of disappearance of AsA in the presence of free copper were usually linear over the greater part of the observed course of the reaction, and were nearly linear over much of the reaction in the presence of copper complex. The meaning of the symbols used throughout this work is as follows:

Cu_f = concentration of free copper in milliatoms per liter.

Cu_t = concentration of total copper in the presence of the complexing compound in milliatoms per liter.

$Cu_b = Cu_t - Cu_f$ = concentration of copper bound to the complexing compound in milliatoms per liter.

$R_f = -\frac{\Delta AsA}{\Delta t}$ = linear rate of disappearance of AsA in millimoles per liter per minute in the presence of free copper alone.

$R_t = -\frac{\Delta AsA}{\Delta t}$ = linear rate of disappearance of AsA in millimoles per liter per minute in the presence of free copper plus the copper bound to the complexing compound.

$R_b = R_t - R_f$ = the rate of disappearance of AsA in the reaction catalyzed by the copper complex.

R_f/Cu_f = rate of disappearance of AsA in the reaction catalyzed by free copper per milliatom of free copper per liter.

R_b/Cu_b = rate of disappearance of AsA in the reaction catalyzed by the copper complex per milliatom of bound copper per liter.

A few of the experiments were made with the previously mentioned (39) sample of malachite that possessed an unusually high solubility. These are indicated by an asterisk.

The results obtained with a number of amino acids, peptides, and imidazole are given in Table IV. The values for R_f/Cu_f for free copper alone are brought together in Table V, and are also given at the head of each subtable.

Manometric measurements were also made for the catalysis of AsA oxidation by copper–glycine complex. The general form of the gas-exchange curves was the same as that observed for the case of free copper alone—there was a net evolution of carbon dioxide only when most of the AsA had disappeared and the point of complete disappearance of AsA corresponded to one molecule of oxygen consumed per molecule of AsA (i.e. to 189 μl per ml of solution).

Figures 4–6 show the rates of disappearance of AsA in the presence of free copper and the copper complexes of glycine, DL-glutamic acid, and β-alanine. Depending on the concentration of catalyst, the pH, and the particular amino acid, the reaction may be of zero order with respect to the concentration of AsA, or the rate may diminish slightly or increase as time goes on.

TABLE V

The influence of pH on the power of free copper to catalyze the rate of disappearance of ascorbic acid

$P_{}$ atm CO_2	pH	Cu_f, mmole per liter	R_f	R_f/Cu_f
0.775	6.360	0.0222	0.0188	0.847
0.775	6.360	0.0316	0.0269	0.851*
0.410	6.636	0.0164	0.0241	1.47
0.400	6.647	0.0161	0.0243	1.51
0.388	6.660	0.0160	0.0244	1.63
0.368	6.684	0.0204	0.0348	1.70*
0.194	6.957	0.0131	0.0294	2.24
0.0485	7.559	0.0103	0.0528	5.12*
0.0485	7.559	0.00908	0.0524	5.77

NOTE: $[HCO_3^-]$: 0.0356 *M*; [NaCl] : 0.178 *M*.

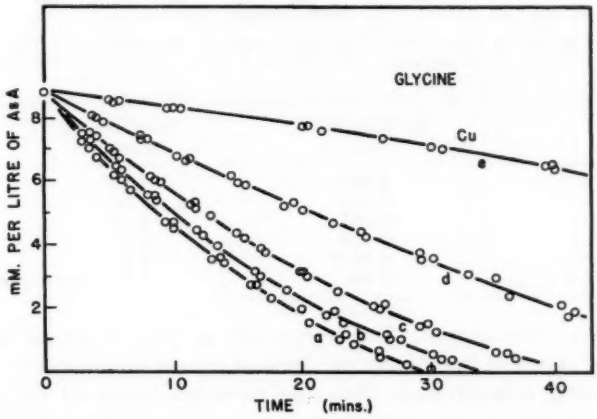

FIG. 4. The rate of disappearance of ascorbic acid in the presence of free copper and copper–glycine complex. HCO_3^-: 0.0356 *M*; NaCl : 0.178 *M*; *P* : 0.0484 atm CO_2.

	Glycine (m*M*)	Bound copper (μg per ml)
a	0.445	9.63
b	0.267	5.26
c	0.178	3.30
d	0.0889	1.38
e	0.00	0.655 (Free copper)

(*b*) *The Effect of Sodium Chloride on the Catalytic Power of Free Copper and Copper Complexes*

It is often stated in the literature that amino acids and proteins are able to prevent or greatly retard the oxidation of AsA in the presence of traces of copper. This effect is usually attributed to the removal of cupric ions from the solution with the formation of an inactive complex. An examination of Tables IV and V, however, reveals that except at pH 7.5, the catalytic power of most of the complexes is often considerably greater than that of an equivalent

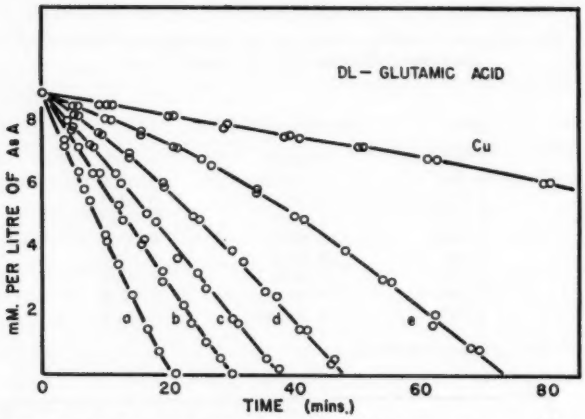

FIG. 5. The rate of disappearance of ascorbic acid in the presence of free copper and the copper complex of DL-glutamic acid. HCO_3: 0.0356 *M*; NaCl : 0.178 *M*; *P* : 0.388 atm CO_2.

	DL-Glutamic (m*M*)	Bound copper (μg per ml)
a	0.889	17.69
b	0.445	7.82
c	0.267	4.70
d	0.178	2.69
e	0.0889	1.72
Cu	0.00	1.33 (Free copper)

concentration of free copper. This is especially so when it is remembered that the catalytic power of free copper per atom of copper diminishes more rapidly with increasing concentration than the catalytic power of the complexes (Table I).

As some of the protective effects reported in the literature were exerted in the absence of sodium chloride, the influence of this salt has been examined in detail. In the first place, bicarbonate buffers containing added copper sulphate and various concentrations of glycine, but no sodium chloride, showed decreased rates of destruction of AsA with increasing concentrations of glycine. There was still an appreciable rate of destruction, however, even in the presence of a final glycine concentration of 8.89 m*M*.

Further experiments were carried out at 10° C. Malachite was equilibrated with 0.04 *M* sodium bicarbonate containing various concentrations of sodium chloride and a constant concentration of glycine (0.5 mmole per liter) at a partial pressure of 0.4 atm carbon dioxide.

The rates of destruction of AsA, together with those for the corresponding solutions containing no added glycine, are compared in Table VI. The net result of the strikingly different effect of sodium chloride on the two catalytic processes is that the catalytic power of free copper is ten times greater than that of copper–glycine in the absence of sodium chloride whereas, when the concentration of sodium chloride is 0.178 *M*, copper–glycine is twice as power-

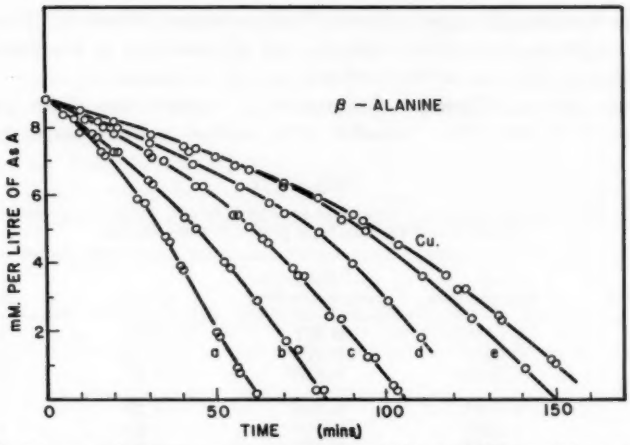

FIG. 6. The rate of disappearance of ascorbic acid in the presence of free copper and the copper complex of β-alanine. HCO_3 : 0.0356 *M*; NaCl : 0.178 *M*; *P* : 0.388 atm CO_2.

	β-alanine (m*M*)	Bound copper (μg per ml)
a	2.667	2.24
b	1.778	1.49
c	0.889	0.89
d	0.445	0.52
e	0.178	0.11
Cu	0.00	1.33 (Free copper)

TABLE VI

Influence of sodium chloride on the rate of disappearance of ascorbic acid in the presence of free copper plus copper–glycine at 10° C

	NaCl, *M*				
	0	0.0445	0.0711	0.0889	0.178
R_f/Cu_f (*a*)	0.295	0.0733	0.0383	0.0200	0.0090
R_b/Cu_b (*b*)	0.0292	0.0424	0.0448	0.0381	0.018
a/*b*	10.1	1.73	0.854	0.525	0.510

NOTE: $[HCO_3^-]$: 0.0356 *M*; [Glycine] : 0.445 m*M*; *P* : 0.410.

ful as free copper. A few experiments carried out at 25° C were sufficient to show a similar differential effect of sodium chloride on the values of R_f/Cu_f and R_b/Cu_b.

(*c*) *The Catalytic Activity of Complexes of Copper with Proteins*

It has already been shown (39) that the three crystallized proteins—bovine plasma albumen, lysozyme, and pepsin—do not bind copper in the same way as does an amino acid at a comparable molarity. The number of bound copper atoms can be reduced by dialysis. It was thought that the more firmly bound atoms of copper might prove to be more powerful catalysts than those more loosely held, but no evidence has been obtained to support this view in the

case of the five bound copper atoms of bovine plasma albumen. Evidence for this was sought by two means: dialysis and the addition of small amounts of copper sulphate directly to the buffered protein solution.

(1) *Bovine plasma albumen.*—The results of experiments of the above type are set out in Table VII. Ascorbic acid oxidase is inactivated during the

TABLE VII

The relation between the amount of copper bound to bovine plasma albumen and the catalytic power of the complex

Treatment	C_a, mmole per liter	Cu_b, mmole per liter	Cu_b/C_a	R_b/Cu_b
e	0.0889	0.451	5.07	0.257
e	0.0445	0.227	5.10	0.316
e	0.00445	0.0231	5.18	0.291
a	0.0445	0.209	4.70	0.283
d	0.0445	0.208	4.67	0.295
a	0.0445	0.171	3.84	0.269

NOTE: e = equilibration; d = dialysis; a = addition of copper sulphate.
During oxidation $[HCO_3^-]$: 0.0356 *M*; [NaCl] : 0.178 *M*; *P*: 0.410; pH : 6.636; R_i/Cu_i : 1.47.

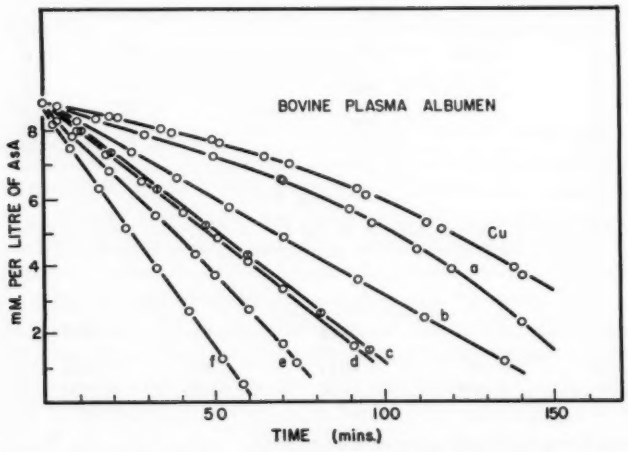

FIG. 7. The oxidation of ascorbic acid in the presence of free copper and the copper complex of bovine plasma albumen, and in the presence of added copper sulphate and the albumen. HCO_3 : 0.0356 *M*; NaCl : 0.178 *M*; *P* : 0.410 atm CO_2.

	Catalysis by the complex			Catalysis by mixtures	
	Albumen (mmole per liter)	Bound copper (μg per ml)		Albumen (mmole per liter)	Added copper (μg Cu per ml)
a	0.00445	1.47	*b*	0.0445	11.31
c	0.0445 (dialyzed complex)	11.7	*d*	0.0445	14.13
e	0.0445	14.43			
f	0.0889	28.65			
Cu (free copper)		1.04			

oxidation of AsA but the copper–albumen complex is apparently unaffected for, if at the end of the reaction the original concentration of AsA is again added, the subsequent rate of destruction of AsA is even slightly higher than the original, presumably due to the presence of peroxides. The rate curves obtained are shown in Fig. 7.

(2) *Pepsin.*—The results obtained by equilibration, dialysis, and addition of copper sulphate to buffered solutions of pepsin are set out in Table VIII. It will be seen that although the pepsin and albumen molecules bind a similar

TABLE VIII

The relation between the amount of copper bound to pepsin and the catalytic power of the complex

Treatment	C_a (mmole per liter)	Cu_b (mmole per liter)	Cu_b/C_a	R_b/Cu_b
e	0.0889	0.396	4.46	0.909
e	0.0445	0.211	4.74	1.16
e	0.0178	0.0838	4.71	1.10
e	0.00889	0.0395	4.45	1.22
e	0.00445	0.0192	4.31	0.953
a	0.0445	0.166	3.72	0.931
d	0.0178	0.0578	3.25	1.12
a	0.0445	0.125	2.82	0.966
d	0.0445	0.115	2.59	1.28
d	0.178	0.0449	2.52	1.22
d	0.00889	0.0188	2.11	1.76
a	0.0445	0.0848	1.91	1.67
a	0.0445	0.0445	1.00	2.29
a	0.0178	0.0178	1.00	2.97

NOTE: During oxidation $[HCO_3^-]$: 0.0356 *M*; [NaCl] : 0.178 *M*; *P* : 0.400; pH : 6.647; R_f/Cu_f : 1.51.

number of copper atoms (4½ against 5), each bound copper atom of the pepsin complex is, on the average, approximately four times more powerful than the average copper atom bound to bovine plasma albumen. Moreover, it appears that when the value of Cu_b/C_a corresponds to one atom of bound copper per molecule of pepsin, the catalytic power of this atom is approximately two and one-half times greater than that of the average catalytic power of the 4½ atoms bound, when the concentration of free copper is that provided by malachite. Pepsin, as mentioned previously (39), tends to be unstable at neutral pH values and some degree of uncertainty thus remains with the results obtained with the dialyzed copper complex. It should be mentioned, however, that when copper sulphate was added directly to pepsin solutions, there was no change in their catalytic power when they were held for much longer periods then those required for the measurement of the rate of destruction of AsA.

(3) *Lysozyme.*—This protein differs from the other two in binding very little copper. Its copper complex, such as it is, has no catalytic power, but on the contrary possesses the striking property of powerfully inhibiting the destruction of AsA in the presence of free copper. The effect is illustrated in Fig. 8. The extent of retardation increased with the concentration of lysozyme present

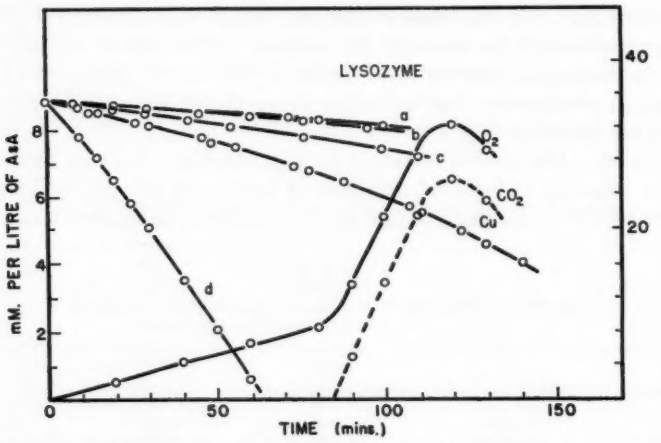

FIG. 8. The inhibition of the oxidation of ascorbic acid by lysozyme–copper complex when catalyzed by free copper and copper-glycine complex. HCO_3 : 0.0356 *M*; NaCl : 0.178 *M*; *P* : 0.400 atm CO_2.
Curves for O_2 absorption and CO_2 evolution correspond to curve *d*.

Disappearance of ascorbic acid

	Lysozyme (mmole per liter)	Bound copper (μg per ml)
a	0.889	2.21
b	0.445	1.10
c	0.0445	0.04
d	0.445 0.445 mmole glycine per liter	9.47
Cu (free copper)		1.03

although all the solutions contained the same amount of free copper. The lysozyme complex also inhibits the catalytic activity of the copper–glycine complex. In this experiment, equal molarities of the protein and the amino acid were equilibrated with malachite, and the catalytic activity of the solution was determined. In the presence of lysozyme, the R_b/Cu_b for copper–glycine under the prevailing conditions is 2.65; in the presence of lysozyme (0.445 mmole per liter) the value of R_b/Cu_b (for the glycine complex) was 0.647. The corresponding rate curves for oxygen absorption and carbon dioxide evolution resemble those observed in the absence of lysozyme in that a net evolution of carbon dioxide was delayed until all the AsA had disappeared. The volume of oxygen finally absorbed, however, was much greater than that occurring in the absence of lysozyme.

Discussion

The results obtained when AsA underwent aerobic oxidation in the presence of dissolved copper alone are in general agreement with those reported in the literature. The disappearance of one molecule of AsA was associated with

the absorption of approximately one molecule of oxygen, and the reaction was greatly retarded by the presence of sodium chloride. The relative catalytic efficiency of the cupric ion and the postulated $CuCO_3$ complex (40) could not be assessed from the present measurements since it was not easy to change their relative concentrations without an alteration of other variables. A similar uncertainty attaches to the use of any buffer that is able to form a complex with copper.

At first sight there seems to be little relation between the affinity of an amino acid for copper and the catalytic power of the complex, especially when comparisons are made at pH 6.66 where there is usually little variation in R_b/Cu_b with Cu_b. Some effect of structure can be traced, but structure has a much greater effect on the avidity with which the compound binds copper. For example, the value of R_b/Cu_b for the complex of DL-α-alanine is only approximately twice as large as the value for the complex of β-alanine, whereas the α-acid under comparable conditions binds nearly twenty times as much copper as the β-acid. Again under comparable conditions, DL-β-phenylalanine binds considerably more copper than glycine yet there is little difference between the activity of the two complexes. Nevertheless, there is a tendency for the more powerful complexing agents to provide the more powerful catalysts; this can be seen when DL-tryptophane and DL-serine are compared with the other compounds studied. At the other end of the scale, β-alanine has the smallest affinity for copper of all the amino acids and provides the weakest catalyst.

It should be remembered that any mechanism which could remove AsA from solution other than by catalyzed aerobic oxidation would invalidate these comparisons. It has been reported (44) that certain cyclic amino acids are able to react with AsA in solution and that this reaction is catalyzed by Cu^{++}. In the present investigations it was noted that high concentrations of the complexes of DL-histidine and histamine caused an initial rapid destruction of AsA in the reaction solution. This effect became increasingly more marked at alkaline pH values. Consequently no data for these complexes have been presented in this work.

The ability to catalyze the oxidation of AsA seems to depend mainly on the local co-ordinating structure in which the copper is bound, rather than on the molecular structure of the complex as a whole.

Molecule for molecule, plasma albumen and pepsin were able to combine with considerably more copper than the glycyl peptides, but the average catalytic power of protein–copper, atom for atom, was less than that of amino acid – copper or peptide–copper. It is interesting to note, however, that copper–protein complexes have been found to be better catalysts than cupric ions for the aerobic oxidation of DOPA (45). Lysozyme was in a different category from the other two proteins. It was hardly able to bind copper itself, yet its complex could retard the destruction of AsA by free copper or a mixture of free copper and copper–glycine. If the differences between the proteins can be attributed to the presence of specific groupings in the protein molecules, it is evident that none of these groupings conferred on the copper bound to

them a catalytic power that in any way resembled that of the copper in ascorbic acid oxidase. The oxidase studied by Lovett-Janison and Nelson (46) had a molecular weight of 150,000 and contained six atoms of copper. This proportion is less than that prevailing in the copper protein–complexes studied (e.g. 36,000 g of pepsin contained approximately 5 g-atoms of copper). A reduction of the copper in pepsin to a proportion of 1 atom per 36,000 g in the expectation that the more firmly bound copper would be more active seemed to show such an effect. The activity of this residual copper, however, was of an entirely different order from that of oxidase copper; the results of Lovett-Janison suggest that the value of R_b/Cu_b for the copper in the enzyme is greater than 2000.

Perhaps the most striking result obtained in the present work on the kinetics of the copper-catalyzed destruction of AsA was that the effect of sodium chloride on the rate in the presence of free copper alone was very different from its effect on the rate in the presence of a mixture of free copper and copper–glycine. The destruction of AsA in the presence of free copper was found to be a first order reaction with respect to the concentration of AsA. An increasing concentration of sodium chloride not only markedly retarded the rate but also changed the order of reaction. At sufficiently high concentrations of sodium chloride, the rate of reaction was either of zero order or increased steadily throughout the entire course of the reaction. In the case of the mixture, that part of the rate attributed to copper–glycine was relatively unaffected, or even increased at some concentrations of sodium chloride. Such results are indicative that the two catalytic mechanisms are independent. It seems probable that the oxidation of AsA in the presence of copper is a chain reaction.

It has been suggested (38) that sodium chloride is an effective inhibitor of the copper-catalyzed oxidation of AsA because it retards one of the reactions involved in the linked reactions Cu^{++}–Cu^{+} and AsA–DHA. Any mechanism involving a net reduction in the concentration of cupric ions in solution caused by the presence of sodium chloride would presumably lead to a dissociation of the copper complex and a fall in the catalytic activity attributed to the complex, the extent of which would be expected to depend on the concentration of sodium chloride. No such effect was observed.

On general grounds, the behavior of sodium chloride (which apparently only inhibits the copper-catalyzed oxidation) and lysozyme (which inhibits the activity of both free copper and copper–glycine) would seem to be more plausibly explained in terms of chain mechanisms than on a basis restricted to direct oxidations and reductions.

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